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**Patents Form 1/77**

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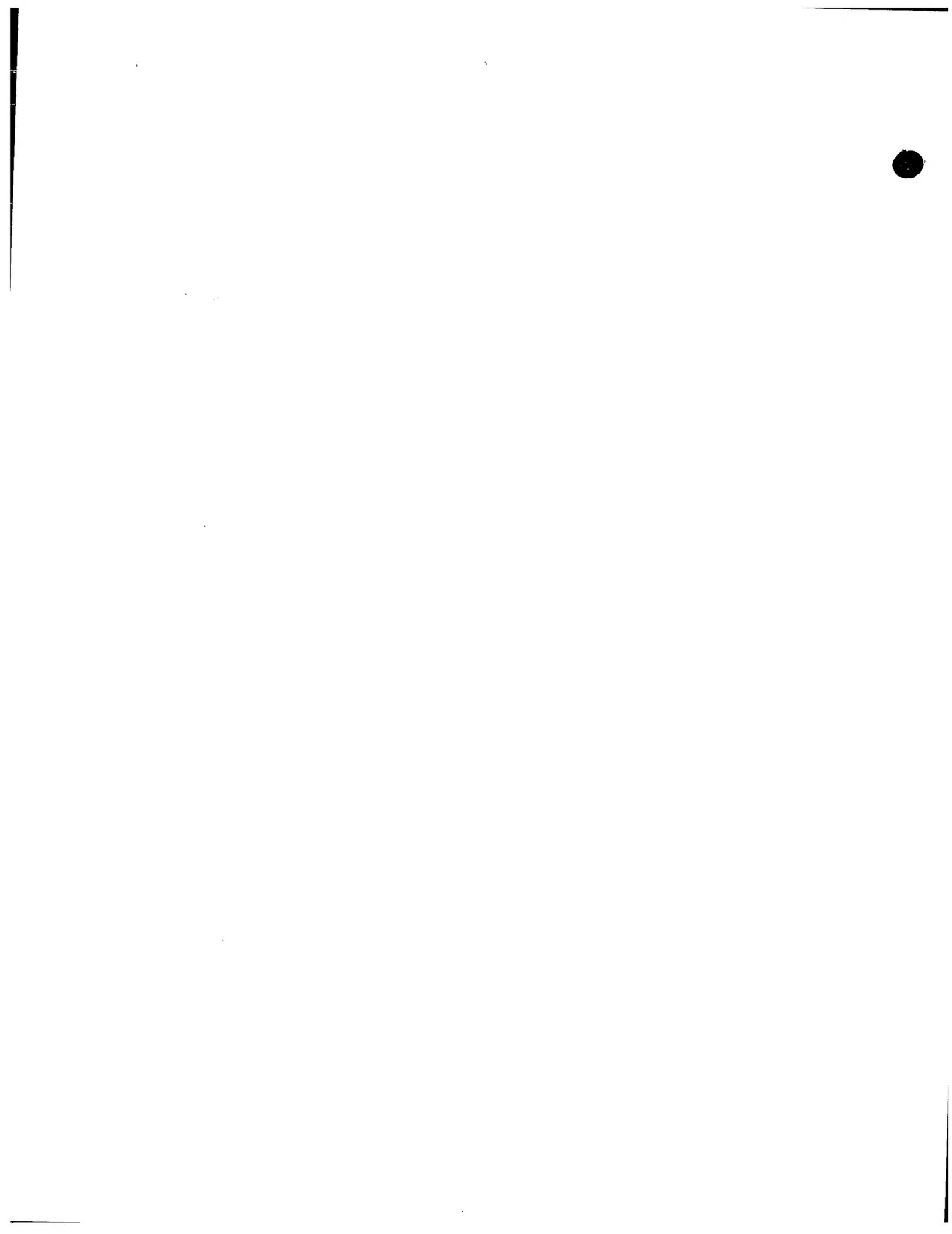
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| 1. | Your reference | P34234-/CMU/MCM |

26NOV03 F855185-1 D02884
P01/7700 0.00-0327493.3

2 6 NOV 2003

| | | |
|---|---|---|
| 2. | Patent application number *(The Patent Office will fill in th)* | **0327493.3** |
| 3. | Full name, address and postcode of the or of each applicant *(underline all surnames)* | The Queen's University of Belfast<br>University Road<br>Belfast<br>BT7 1NN |
| | Patents ADP number *(if you know it)* | 8896059006 |
| | If the applicant is a corporate body, give the country/state of its incorporation | United Kingdom |
| 4. | Title of the invention | "Treatment Medicament" |
| 5. | Name of your agent *(if you have one)* | Murgitroyd & Company |
| | "Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)* | Scotland House<br>165-169 Scotland Street<br>Glasgow<br>G5 8PL |
| | Patents ADP number *(if you know it)* | 1198013 |

| | | Country | Priority application number *(if you know it)* | Date of filing *(day / month / year)* |
|---|---|---|---|---|
| 6. | If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number | | | |

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| 7. | If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application | | | |

| | | |
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| 8. | Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if* <br> a) *any applicant named in part 3 is not an inventor, or* <br> b) *there is an inventor who is not named as an applicant, or* <br> c) *any named applicant is a corporate body.* <br> *See note (d))* | Yes |

**Patents Form 1/77**

## Patents Form 1/77

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   Description                          59

   Claim(s)                             5

   Abstract                             -

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    Priority documents

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    Statement of inventorship and right
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    Request for preliminary examination
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    Request for substantive examination
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    Any other documents
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11.

I/We request the grant of a patent on the basis of this application.

Signature *Murgitroyd & Co*

Murgitroyd & Company

Date
26 November 2003

12. Name and daytime telephone number of
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## Patents Form 1/77

1

1                      **"Treatment Medicament"**

2

3     **Field of the Invention**

4

5     This application relates to a medicament and its use

6     in methods of treatment. In particular, it relates

7     to the treatment of cancer with a death receptor

8     ligand, e.g. a FAS (CD95 or TNF receptor 2) receptor

9     ligand, and a chemotherapeutic agent.

10

11     **Background to the Invention**

12

13     Breast, oesophageal, colorectal, all forms of GI

14     cancer and head and neck cancers are highly

15     malignant with overall 5-year survival rates of less

16     than 50%. The clinical outcome of these patients is

17     predetermined by the presence of widely disseminated

18     tumour cells termed micrometastases with potential

19     for metastatic growth, prior to clinical

20     presentation. Approximately 50% of oesophageal

21     cancer patients are selected for surgical therapy

1    with 30% 5-year survival for this patient sub-group.

2    Randomised clinical trials of neoadjuvant 5FU-based

3    chemotherapy combined with fractionated radiotherapy

4    have demonstrated improvements in survival of 10-

5    20%, although the overall 5-year outcome for the

6    treated groups remains at 30-35%.  Those patients

7    who demonstrate complete pathological response in

8    their primary tumours as a result of neoadjuvant

9    treatment have a five-year survival of 80%.

10   Conversely, those patients who do not respond to

11   5FU-based chemotherapy are denied the opportunity

12   for earlier treatment by surgery or a different

13   neoadjuvant chemotherapeutic based regimen. Thus,

14   there is an urgent need for improved therapeutic

15   strategies.

16

17   5-FU[1] is widely used in the treatment of a range of

18   cancers including colorectal, breast and cancers of

19   the aerodigestive tract. The mechanism of

20   cytotoxicity of 5-FU has been ascribed to the

21   misincorporation of fluoronucleotides into RNA and

22   DNA and to the inhibition of the nucleotide

23   synthetic enzyme thymidylate synthase (TS) (1). TS

24   catalyses the conversion of deoxyuridine

25   monophosphate (dUMP) to deoxythymidine monophosphate

26   (dTMP) with 5,10-methylene tetrahydrofolate ($CH_2THF$)

27   as the methyl donor. This reaction provides the sole

28   intracellular source of thymidylate, which is

29   essential for DNA synthesis and repair. The 5-FU

30   metabolite fluorodeoxyuridine monophosphate (FdUMP)

31   forms a stable complex with TS and $CH_2THF$ resulting

32   in enzyme inhibition (1). Recently, more specific

3

1  folate-based inhibitors of TS have been developed

2  such as RTX and MTA, which form a stable complex

3  with TS and dUMP that inhibits binding of $CH_2THF$ to

4  the enzyme (2, 3). TS inhibition causes nucleotide

5  pool imbalances that result in S phase cell cycle

6  arrest and apoptosis (4-6).

7

8

9  **Summary of the Invention**

10

11  As described herein, the present inventors have

12  surprisingly shown that by combining treatment using

13  a death receptor ligand, such as an anti FAS

14  antibody, with a chemotherapeutic agent such as 5-FU

15  or an antifolate drug, such as ralitrexed (RTX) or

16  pemetrexed (MTA, Alimta), a synergistic effect is

17  achieved in the killing of cancer cells.

18

19  Accordingly, in a first aspect, the present

20  invention provides a method of killing cancer cells

21  comprising administration of a therapeutically

22  effective amount of a) a specific binding member

23  which binds to a cell death receptor or a nucleic

24  acid encoding said binding member and (b) a

25  chemotherapeutic agent.

26

27  In a second aspect, the present invention provides a

28  method of treating cancer comprising administration

29  of a therapeutically effective amount of a) a

30  specific binding member which binds to a cell death

31  receptor or a nucleic acid encoding said binding

4

1       member and (b) a chemotherapeutic agent to a mammal

2       in need thereof.

3

4       The specific binding member and the chemotherapeutic

5       agent may be administered simultaneously,

6       sequentially or simultaneously. In preferred

7       embodiments of the invention, the chemotherapeutic

8       agent is administered prior to the specific binding

9       member.

10

11      In a third aspect, there is provided the use of (a)

12      a specific binding member which binds to a cell

13      death receptor or a nucleic acid encoding said

14      binding member and (b) a chemotherapeutic agent in

15      the preparation of a medicament for treating cancer.

16

17      In a fourth aspect, there is provided a product

18      comprising a) a specific binding member which binds

19      to a cell death receptor or a nucleic acid encoding

20      said binding member and (b) a chemotherapeutic agent

21      as a combined preparation for the simultaneous,

22      separate or sequential use in the treatment of

23      cancer.

24

25      According to a fifth aspect, there is provided a

26      pharmaceutical composition for the treatment of

27      cancer, wherein the composition comprises a) a

28      specific binding member which binds to a cell death

29      receptor or a nucleic acid encoding said binding

30      member and (b) a chemotherapeutic agent and (c) a

31      pharmaceutically acceptable excipient, diluent or

5

1   carrier.

2

3   In a sixth aspect, there is provided a kit for the

4   treatment of cancer, said kit comprising a) a

5   specific binding member which binds to a cell death

6   receptor or a nucleic acid encoding said binding

7   member and (b) a chemotherapeutic agent and (c)

8   instructions for the administration of (a) and (b)

9   separately, sequentially or simultaneously.

10

11  The invention may be used to treat any cancer.   In

12  preferred embodiments of the invention, the cancer

13  is one or more of colorectal, breast , ovarian,

14  cervical, gastric, lung, liver, skin and myeloid

15  (e.g. bone marrow) cancer.

16

17  In preferred embodiments of the invention, the

18  binding member is an antibody or a fragment thereof.

19  In particularly preferred embodiments, the binding

20  member is the FAS antibody CH11 (Yonehara, S.,

21  Ishii, A. and Yonehara, M. (1989) J. Exp. Med. 169,

22  1747-1756) (available commercially e.g. from Upstate

23  Biotechnology, Lake Placid, NY).

24

25  The binding member may bind to any death receptor.

26  Death receptors include, Fas, TNFR, DR-3, DR-4 and

27  DR-5. In preferred embodiments of the invention, the

28  death receptor is FAS.

29

30  In preferred embodiments, the binding member

31  comprises at least one human constant region.

32

6

1    Any suitable chemotherapeutic agent may be used in

2    the present invention. In preferred embodiments, the

3    agent is doxorubicin, oxaliplatin, taxol, tomudex

4    (TDX), 5-Fluorouracil (5-FU), Irinotecan (CPT11) or

5    an antifolate e.g. MTA or RTX.  In one preferred

6    embodiment, the agent is tomudex, 5-Fluorouracil, an

7    antifolate (for example RTX or MTA), or a

8    combination thereof.  In a particularly preferred

9    embodiment, the agent is 5-FU or an antifolate. In

10    another preferred embodiment, the agent is an

11    antifolate. In a particularly preferred embodiment

12    the agent is MTA.

13

14    The invention also provides a method of treating

15    tumour cells, the method including the steps of

16    administering a compound capable of triggering or

17    binding a death receptor, e.g. a binding member and

18    administering a chemotherapeutic agent.

19

20    The concentrations of binding members and

21    chemotherapeutic agents used are preferably

22    sufficient to provide a synergistic effect.

23    Synergism is preferably defined as an RI of greater

24    than unity using the method of Kern as modified by

25    Romaneli (13, 14). The RI may be calculated as the

26    ratio of expected cell survival ($S_{exp}$, defined as the

27    product of the survival observed with drug A alone

28    and the survival observed with drug B alone) to the

29    observed cell survival ($S_{obs}$) for the combination of

30    A and B ($RI=S_{exp}/S_{obs}$). Synergism may then be defined

31    as an RI of greater than unity.

32

7

1    In preferred embodiments of the invention, said
2    specific binding member and chemotherapeutic agent
3    are provided in concentrations sufficient to produce
4    an RI of greater than 1.5, more preferably greater
5    than 2.0, most preferably greater than 2.25.
6
7    The combined medicament thus preferably produces a
8    synergistic effect when used to treat tumour cells.
9
10   A seventh aspect of the present invention therefore
11   provides a medicament for use in treating tumour
12   cells, the medicament comprising at least one
13   antibody directed at FAS receptor and at least one
14   cancer chemotherapeutic agent.
15
16   The invention also provides in a eighth aspect a
17   method of treating tumour cells, the method
18   including the steps of administering a compound
19   capable of triggering or binding a death receptor
20   and administering simultaneously, sequentially or
21   separately a chemotherapeutic agent.
22
23   In an ninth aspect, the invention provides the use
24   of an antibody directed at FAS receptor in
25   combination with a cancer chemotherapeutic agent in
26   the preparation of a medicament for treatment of
27   tumour cells.
28
29   In a particular aspect, the application relates to
30   the use of an antibody or a fas ligand directed at a
31   death receptor e.g. the FAS receptor (CD95/TNF
32   receptor 2) to synergise with cancer

8

1    chemotherapeutic agents, e.g. 5-FU or an antifolate,

2    for example RTX or MTA, to enhance therapy and

3    enhance the removal or regression of tumour cells.

4

5    This application is relevant for, but is not limited

6    to, breast cancer, oesophageal cancer, colorectal

7    cancer, all forms of GI cancer and head and neck

8    cancers and may also be used to target other cells

9    via death receptors.

10

11    Preferred features of each aspect of the invention

12    are as for each of the other aspects mutatis

13    mutandis.

14

15    **Detailed Description**

16

17    **Binding members**

18

19    In the context of the present invention, a "binding

20    member" is a molecule which has binding specificity

21    for another molecule, in particular a receptor, in

22    particular a death receptor. The binding member may

23    be a member of a pair of specific binding members.

24    The members of a binding pair may be naturally

25    derived or wholly or partially synthetically

26    produced. One member of the pair of molecules may

27    have an area on its surface, which may be a

28    protrusion or a cavity, which specifically binds to

29    and is therefore complementary to a particular

30    spatial and polar organisation of the other member

31    of the pair of molecules. Thus, the members of the

32    pair have the property of binding specifically to

1   each other.  Examples of types of binding pairs are
2   antigen-antibody, biotin-avidin, hormone-hormone
3   receptor, receptor-ligand, enzyme-substrate.  A
4   binding member of the invention and for use in the
5   invention may be any moiety, for example an antibody
6   or ligand, which can bind to a death receptor.
7
8   **Antibodies**
9
10  An "antibody" is an immunoglobulin, whether natural
11  or partly or wholly synthetically produced.  The
12  term also covers any polypeptide, protein or peptide
13  having a binding domain which is, or is homologous
14  to, an antibody binding domain.  These can be
15  derived from natural sources, or they may be partly
16  or wholly synthetically produced.  Examples of
17  antibodies are the immunoglobulin isotypes and their
18  isotypic subclasses and fragments which comprise an
19  antigen binding domain such as Fab, scFv, Fv, dAb,
20  Fd; and diabodies.
21
22  The binding member of the invention may be an
23  antibody such as a monoclonal or polyclonal
24  antibody, or a fragment thereof. The constant region
25  of the antibody may be of any class including, but
26  not limited to, human classes IgG, IgA, IgM, IgD and
27  IgE.  The antibody may belong to any sub class e.g.
28  IgG1, IgG2, IgG3 and IgG4.  IgG1 is preferred.
29
30  As antibodies can be modified in a number of ways,
31  the term "antibody" should be construed as covering
32  any binding member or substance having a binding

10

1    domain with the required specificity.   Thus, this

2    term covers antibody fragments, derivatives,

3    functional equivalents and homologues of antibodies,

4    including any polypeptide comprising an

5    immunoglobulin binding domain, whether natural or

6    wholly or partially synthetic.   Chimeric molecules

7    comprising an immunoglobulin binding domain, or

8    equivalent, fused to another polypeptide are

9    therefore included.   Cloning and expression of

10   chimeric antibodies are described in EP-A-0120694

11   and EP-A-0125023.

12

13   It has been shown that fragments of a whole antibody

14   can perform the function of binding antigens.

15   Examples of such binding fragments are (i) the Fab

16   fragment consisting of VL, VH, CL and CH1 domains;

17   (ii) the Fd fragment consisting of the VH and CH1

18   domains; (iii) the Fv fragment consisting of the VL

19   and VH domains of a single antibody; (iv) the dAb

20   fragment (Ward, E.S. et al., Nature 341:544-546

21   (1989)) which consists of a VH domain; (v) isolated

22   CDR regions; (vi) F(ab')2 fragments, a bivalent

23   fragment comprising two linked Fab fragments (vii)

24   single chain Fv molecules (scFv), wherein a VH

25   domain and a VL domain are linked by a peptide

26   linker which allows the two domains to associate to

27   form an antigen binding site (Bird et al., Science

28   242:423-426 (1988); Huston et al., PNAS USA 85:5879-

29   5883 (1988)); (viii) bispecific single chain Fv

30   dimers (PCT/US92/09965) and (ix) "diabodies",

31   multivalent or multispecific fragments constructed

11

1   by gene fusion (WO94/13804; P. Hollinger et al.,

2   Proc. Natl. Acad. Sci. USA 90:6444-6448 (1993)).

3

4   A fragment of an antibody or of a polypeptide for

5   use in the present invention generally means a

6   stretch of amino acid residues of at least 5 to 7

7   contiguous amino acids, often at least about 7 to 9

8   contiguous amino acids, typically at least about 9

9   to 13 contiguous amino acids, more preferably at

10   least about 20 to 30 or more contiguous amino acids

11   and most preferably at least about 30 to 40 or more

12   consecutive amino acids.

13

14   A "derivative" of such an antibody or polypeptide,

15   or of a fragment antibody means an antibody or

16   polypeptide modified by varying the amino acid

17   sequence of the protein, e.g. by manipulation of the

18   nucleic acid encoding the protein or by altering the

19   protein itself. Such derivatives of the natural

20   amino acid sequence may involve insertion, addition,

21   deletion and/or substitution of one or more amino

22   acids, preferably while providing a peptide having

23   death receptor, e.g. FAS neutralisation and/or

24   binding activity. Preferably such derivatives

25   involve the insertion, addition, deletion and/or

26   substitution of 25 or fewer amino acids, more

27   preferably of 15 or fewer, even more preferably of

28   10 or fewer, more preferably still of 4 or fewer and

29   most preferably of 1 or 2 amino acids only.

30

31   The term "antibody" includes antibodies which have

32   been "humanised". Methods for making humanised

1    antibodies are known in the art.  Methods are

2    described, for example, in Winter, U.S. Patent No.

3    5,225,539.  A humanised antibody may be a modified

4    antibody having the hypervariable region of a

5    monoclonal antibody and the constant region of a

6    human antibody.  Thus the binding member may

7    comprise a human constant region.

8

9    The variable region other than the hypervariable

10   region may also be derived from the variable region

11   of a human antibody and/or may also be derived from

12   a monoclonal antibody.  In such case, the entire

13   variable region may be derived from murine

14   monoclonal antibody and the antibody is said to be

15   chimerised.  Methods for making chimerised

16   antibodies are known in the art.  Such methods

17   include, for example, those described in U.S.

18   patents by Boss (Celltech) and by Cabilly

19   (Genentech).  See U.S. Patent Nos. 4,816,397 and

20   4,816,567, respectively.

21

22   It is possible to take monoclonal and other

23   antibodies and use techniques of recombinant DNA

24   technology to produce other antibodies or chimeric

25   molecules which retain the specificity of the

26   original antibody.  Such techniques may involve

27   introducing DNA encoding the immunoglobulin variable

28   region, or the complementary determining regions

29   (CDRs), of an antibody to the constant regions, or

30   constant regions plus framework regions, of a

31   different immunoglobulin.  See, for instance, EP-A-

32   184187, GB 2188638A or EP-A-239400.  A hybridoma or

13

1  other cell producing an antibody may be subject to

2  genetic mutation or other changes, which may or may

3  not alter the binding specificity of antibodies

4  produced.

5

6  A typical antibody for use in the present invention

7  is a humanised equivalent of CH11 or any chimerised

8  equivalent of an antibody that can bind to the FAS

9  receptor and any alternative antibodies directed at

10  the FAS receptor that have been chimerised and can

11  be use in the treatment of humans. Furthermore, the

12  typical antibody is any antibody that can cross-

13  react with the extracellular portion of the FAS

14  receptor and either bind with high affinity to the

15  FAS receptor, be internalised with the FAS receptor

16  or trigger signalling through the FAS receptor.

17

18  **Production of Binding Members**

19

20  The binding members for use in the present invention

21  may be generated wholly or partly by chemical

22  synthesis.  The binding members can be readily

23  prepared according to well-established, standard

24  liquid or, preferably, solid-phase peptide synthesis

25  methods, general descriptions of which are broadly

26  available (see, for example, in J.M. Stewart and

27  J.D. Young, Solid Phase Peptide Synthesis, 2nd

28  edition, Pierce Chemical Company, Rockford, Illinois

29  (1984), in M. Bodanzsky and A. Bodanzsky, The

30  Practice of Peptide Synthesis, Springer Verlag, New

31  York (1984); and Applied Biosystems 430A Users

32  Manual, ABI Inc., Foster City, California), or they

14

1    may be prepared in solution, by the liquid phase

2    method or by any combination of solid-phase, liquid

3    phase and solution chemistry, e.g. by first

4    completing the respective peptide portion and then,

5    if desired and appropriate, after removal of any

6    protecting groups being present, by introduction of

7    the residue X by reaction of the respective carbonic

8    or sulfonic acid or a reactive derivative thereof.

9

10   Another convenient way of producing a binding member

11   suitable for use in the present invention is to

12   express nucleic acid encoding it, by use of nucleic

13   acid in an expression system. Thus the present

14   invention further provides the use of (a) nucleic

15   acid encoding a specific binding member which binds

16   to a cell death receptor and (b) a chemotherapeutic

17   agent in the preparation of a medicament for

18   treating cancer.

19

20   Nucleic acid for use in accordance with the present

21   invention may comprise DNA or RNA and may be wholly

22   or partially synthetic. In a preferred aspect,

23   nucleic acid for use in the invention codes for a

24   binding member of the invention as defined above.

25   The skilled person will be able to determine

26   substitutions, deletions and/or additions to such

27   nucleic acids which will still provide a binding

28   member suitable for use in the present invention.

29

30   Nucleic acid sequences encoding a binding member for

31   use with the present invention can be readily

32   prepared by the skilled person using the information

15

1  and references contained herein and techniques known

2  in the art (for example, see Sambrook, Fritsch and

3  Maniatis, "Molecular Cloning", A Laboratory Manual,

4  Cold Spring Harbor Laboratory Press, 1989, and

5  Ausubel et al, Short Protocols in Molecular Biology,

6  John Wiley and Sons, 1992), given the nucleic acid

7  sequences and clones available.  These techniques

8  include (i) the use of the polymerase chain reaction

9  (PCR) to amplify samples of such nucleic acid, e.g.

10  from genomic sources, (ii) chemical synthesis, or

11  (iii) preparing cDNA sequences.  DNA encoding

12  antibody fragments may be generated and used in any

13  suitable way known to those of skill in the art,

14  including by taking encoding DNA, identifying

15  suitable restriction enzyme recognition sites either

16  side of the portion to be expressed, and cutting out

17  said portion from the DNA.  The portion may then be

18  operably linked to a suitable promoter in a standard

19  commercially available expression system.  Another

20  recombinant approach is to amplify the relevant

21  portion of the DNA with suitable PCR primers.

22  Modifications to the sequences can be made, e.g.

23  using site directed mutagenesis, to lead to the

24  expression of modified peptide or to take account of

25  codon preferences in the host cells used to express

26  the nucleic acid.

27

28  The nucleic acid may be comprised as construct(s) in

29  the form of a plasmid, vector, transcription or

30  expression cassette which comprises at least one

31  nucleic acid as described above.  The construct may

32  be comprised within a recombinant host cell which

1     comprises one or more constructs as above.

2     Expression may conveniently be achieved by culturing

3     under appropriate conditions recombinant host cells

4     containing the nucleic acid. Following production

5     by expression a specific binding member may be

6     isolated and/or purified using any suitable

7     technique, then used as appropriate.

8

9     Binding members-encoding nucleic acid molecules and

10    vectors for use in accordance with the present

11    invention may be provided isolated and/or purified,

12    e.g. from their natural environment, in

13    substantially pure or homogeneous form, or, in the

14    case of nucleic acid, free or substantially free of

15    nucleic acid or genes origin other than the sequence

16    encoding a polypeptide with the required function.

17

18    Systems for cloning and expression of a polypeptide

19    in a variety of different host cells are well known.

20    Suitable host cells include bacteria, mammalian

21    cells, yeast and baculovirus systems. Mammalian

22    cell lines available in the art for expression of a

23    heterologous polypeptide include Chinese hamster

24    ovary cells, HeLa cells, baby hamster kidney cells,

25    NSO mouse melanoma cells and many others. A common,

26    preferred bacterial host is E. coli.

27

28    The expression of antibodies and antibody fragments

29    in prokaryotic cells such as E. coli is well

30    established in the art. For a review, see for

31    example Plückthun, Bio/Technology 9:545-551 (1991).

32    Expression in eukaryotic cells in culture is also

17

1    available to those skilled in the art as an option

2    for production of a binding member, see for recent

3    review, for example Reff, Curr. Opinion Biotech.

4    4:573-576 (1993); Trill et al., Curr. Opinion

5    Biotech. 6:553-560 (1995).

6

7    Suitable vectors can be chosen or constructed,

8    containing appropriate regulatory sequences,

9    including promoter sequences, terminator sequences,

10   polyadenylation sequences, enhancer sequences,

11   marker genes and other sequences as appropriate.

12   Vectors may be plasmids, viral e.g. 'phage, or

13   phagemid, as appropriate. For further details see,

14   for example, Sambrook et al., Molecular Cloning: A

15   Laboratory Manual: 2nd Edition, Cold Spring Harbor

16   Laboratory Press (1989). Many known techniques and

17   protocols for manipulation of nucleic acid, for

18   example in preparation of nucleic acid constructs,

19   mutagenesis, sequencing, introduction of DNA into

20   cells and gene expression, and analysis of proteins,

21   are described in detail in Ausubel et al. eds.,

22   Short Protocols in Molecular Biology, 2nd Edition,

23   John Wiley & Sons (1992).

24

25   The nucleic acid may be introduced into a host cell

26   by any suitable means. The introduction may employ

27   any available technique. For eukaryotic cells,

28   suitable techniques may include calcium phosphate

29   transfection, DEAE-Dextran, electroporation,

30   liposome-mediated transfection and transduction

31   using retrovirus or other virus, e.g. vaccinia or,

32   for insect cells, baculovirus. For bacterial cells,

18

1     suitable techniques may include calcium chloride

2     transformation, electroporation and transfection

3     using bacteriophage.

4

5     Marker genes such as antibiotic resistance or

6     sensitivity genes may be used in identifying clones

7     containing nucleic acid of interest, as is well

8     known in the art.

9

10    The introduction may be followed by causing or

11    allowing expression from the nucleic acid, e.g. by

12    culturing host cells under conditions for expression

13    of the gene.

14

15    ·The nucleic acid may be integrated into the genome

16    (e.g. chromosome) of the host cell. Integration may

17    be promoted by inclusion of sequences which promote

18    recombination with the genome in accordance with

19    standard techniques. The nucleic acid may be on an

20    extra-chromosomal vector within the cell, or

21    otherwise identifiably heterologous or foreign to

22    the cell.

23

24    **Chemotherapeutic Agents**

25

26    As described above, the present invention is based

27    on the surprising demonstration that combining

28    treatment using a death receptor ligand such as the

29    CH11 antibody with a chemotherapeutic agent results

30    in a surprisingly enhanced synergistic therapeutic

31    effect.

32

1     Any suitable chemotherapeutic agent or agents may be
2     used in the present invention. For example, the
3     agent for use in the invention may include but is
4     not limited to: 5-Fluorouracil (5 FU), tomudex (TDX)
5     antifolates, for example RTX or MTA, Doxorubicin,
6     taxol, Leucovorin, Irinotecan, Mitomycin C,
7     Oxaliplatin, Raltitrexed, Tamoxifen or Cisplatin.
8

9     In particularly preferred embodiments, the agent is
10    5-FU or an antifolate. More preferably, the agent
11    is an antifolate. In one preferred embodiment, the
12    agent is MTA.
13

14    **Treatment**
15    Treatment" includes any regime that can benefit a
16    human or non-human animal. The treatment may be in
17    respect of an existing condition or may be
18    prophylactic (preventative treatment). Treatment may
19    include curative, alleviation or prophylactic
20    effects.
21

22    "Treatment of cancer" includes treatment of
23    conditions caused by cancerous growth and includes
24    the treatment of neoplastic growths or tumours.
25    Examples of tumours that can be treated using the
26    invention are, for instance, sarcomas, including
27    osteogenic and soft tissue sarcomas, carcinomas,
28    e.g., breast-, lung-, bladder-, thyroid-, prostate-,
29    colon-, rectum-, pancreas-, stomach-, liver-,
30    uterine-, cervical and ovarian carcinoma, lymphomas,
31    including Hodgkin and non-Hodgkin lymphomas,
32    neuroblastoma, melanoma, myeloma, Wilms tumor, and

20

1    leukemias, including acute lymphoblastic leukaemia
2    and acute myeloblastic leukaemia, gliomas and
3    retinoblastomas.
4
5
6    The compositions and methods of the invention may be
7    particularly useful in the treatment of existing
8    cancer and in the prevention of the recurrence of
9    cancer after initial treatment or surgery.
10
11   Administration
12
13   Binding members and chemotherapeutic agents  may be
14   administered simultaneously, separately or
15   sequentially.
16
17   Where administered separately or sequentially, they
18   may be administered within any suitable time period
19   e.g. within 1, 2, 3, 6, 12, 24, 48 or 72 hours of
20   each other.  In preferred embodiments, they are
21   administered within 6, preferably within 2, more
22   preferably within 1, most preferably within 20
23   minutes of each other. ***Please advise on preferred
24   .ranges***
25
26   In a preferred embodiment, they are administered as
27   a pharmaceutical composition, which will generally
28 . comprise a suitable pharmaceutical excipient,
29   diluent or carrier selected dependent on the
30   intended route of administration.
31

21

1   Binding members and chemotherapeutic agents of and
2   for use in the present invention may be administered
3   to a patient in need of treatment via any suitable
4   route. The precise dose will depend upon a number of
5   factors, including the precise nature of the member
6   (e.g. whole antibody, fragment or diabody) and
7   chemotherapeutic agent.
8
9   Some suitable routes of administration include (but
10  are not limited to) oral, rectal, nasal, topical
11  (including buccal and sublingual), vaginal or
12  parenteral (including subcutaneous, intramuscular,
13  intravenous, intradermal, intrathecal and epidural)
14  administration. Intravenous administration is
15  preferred.
16
17  It is envisaged that injections (intravenous) will
18  be the primary route for therapeutic administration
19  of compositions although delivery through a catheter
20  or other surgical tubing is also envisaged.  Liquid
21  formulations may be utilised after reconstitution
22  from powder formulations.
23
24  For intravenous, injection, or injection at the site
25  of affliction, the active ingredient will be in the
26  form of a parenterally acceptable aqueous solution
27  which is pyrogen-free and has suitable pH,
28  isotonicity and stability.  Those of relevant skill
29  in the art are well able to prepare suitable
30  solutions using, for example, isotonic vehicles such
31  as Sodium Chloride Injection, Ringer's Injection,
32  Lactated Ringer's Injection.  Preservatives,

1    stabilisers, buffers, antioxidants and/or other
2    additives may be included, as required.
3
4    Pharmaceutical compositions for oral administration
5    may be in tablet, capsule, powder or liquid form.  A
6    tablet may comprise a solid carrier such as gelatin
7    or an adjuvant.  Liquid pharmaceutical compositions
8    generally comprise a liquid carrier such as water,
9    petroleum, animal or vegetable oils, mineral oil or
10   synthetic oil.  Physiological saline solution,
11   dextrose or other saccharide solution or glycols
12   such as ethylene glycol, propylene glycol or
13   polyethylene glycol may be included.
14
15   The binding member, agent, product or composition
16   may also be administered via microspheres,
17   liposomes, other microparticulate delivery systems
18   or sustained release formulations placed in certain
19   tissues including blood.  Suitable examples of
20   sustained release carriers include semipermeable
21   polymer matrices in the form of shared articles,
22   e.g. suppositories or microcapsules.  Implantable or
23   microcapsular sustained release matrices include
24   polylactides (US Patent No. 3, 773, 919; EP-A-
25   0058481) copolymers of L-glutamic acid and gamma
26   ethyl-L-glutamate (Sidman et al, Biopolymers 22(1):
27   547-556, 1985), poly (2-hydroxyethyl-methacrylate)
28   or ethylene vinyl acetate (Langer et al, J. Biomed.
29   Mater. Res. 15: 167-277, 1981, and Langer, Chem.
30   Tech. 12:98-105, 1982).  Liposomes containing the
31   polypeptides are prepared by well-known methods: DE
32   3,218, 121A; Epstein et al, PNAS USA, 82: 3688-3692,

1   1985; Hwang et al, PNAS USA, 77: 4030-4034, 1980;

2   EP-A-0052522; E-A-0036676; EP-A-0088046; EP-A-

3   0143949; EP-A-0142541; JP-A-83-11808; US Patent Nos

4   4,485,045 and 4,544,545.  Ordinarily, the liposomes

5   are of the small (about 200-800 Angstroms)

6   unilamellar type in which the lipid content is

7   greater than about 30 mol. % cholesterol, the

8   selected proportion being adjusted for the optimal

9   rate of the polypeptide leakage.

10

11  Examples of the techniques and protocols mentioned

12  above and other techniques and protocols which may

13  be used in accordance with the invention can be

14  found in Remington's Pharmaceutical Sciences, 16th

15  edition, Oslo, A. (ed), 1980.

16

17  The binding member, agent, product or composition

18  may be administered in a localised manner to a

19  tumour site or other desired site or may be

20  delivered in a manner in which it targets tumour or

21  other cells. Targeting therapies may be used to

22  deliver the active agents more specifically to

23  certain types of cell, by the use of targeting

24  systems such as antibody or cell specific ligands.

25  Targeting may be desirable for a variety of reasons,

26  for example if the agent is unacceptably toxic, or

27  if it would otherwise require too high a dosage, or

28  if it would not otherwise be able to enter the

29  target cells.

30

31  **Pharmaceutical Compositions**

32

24

1    As described above, the present invention extends to
2    a pharmaceutical composition for the treatment of
3    cancer, the composition comprising a) a specific
4    binding member which binds to a cell death receptor
5    or a nucleic acid encoding said binding member and
6    (b) a chemotherapeutic agent and (c) a
7    pharmaceutically acceptable excipient, diluent or
8    carrier. Pharmaceutical compositions according to
9    the present invention, and for use in accordance
10   with the present invention may comprise, in addition
11   to active ingredients, a pharmaceutically acceptable
12   excipient, carrier, buffer stabiliser or other
13   materials well known to those skilled in the art.
14   Such materials should be non-toxic and should not
15   interfere with the efficacy of the active
16   ingredient.  The precise nature of the carrier or
17   other material will depend on the route of
18   administration, which may be oral, or by injection,
19   e.g. intravenous.
20

21   The formulation may be a liquid, for example, a
22   physiologic salt solution containing non-phosphate
23   buffer at pH 6.8-7.6, or a lyophilised powder.
24

25   Dose
26

27   The binding members, agents, products or
28   compositions are preferably administered to an
29   individual in a "therapeutically effective amount",
30   this being sufficient to show benefit to the
31   individual.  The actual amount administered, and
32   rate and time-course of administration, will depend

1   on the nature and severity of what is being treated.

2   As described herein, the concentrations are

3   preferably sufficient to show a synergistic effect.

4   Prescription of treatment, e.g. decisions on dosage

5   etc, is ultimately within the responsibility and at

6   the discretion of general practitioners and other

7   medical doctors, and typically takes account of the

8   disorder to be treated, the condition of the

9   individual patient, the site of delivery, the method

10   of administration and other factors known to

11   practitioners.

12

13   The optimal dose can be determined by physicians

14   based on a number of parameters including, for

15   example, age, sex, weight, severity of the condition

16   being treated, the active ingredient being

17   administered and the route of administration. For

18   example, with respect to binding members, in

19   general, a serum concentration of polypeptides and

20   antibodies that permits saturation of receptors is

21   desirable.  A concentration in excess of

22   approximately 0.1nM is normally sufficient.  For

23   example, a dose of $100mg/m^2$ of antibody provides a

24   serum concentration of approximately 20nM for

25   approximately eight days.

26

27   As a rough guideline, doses of antibodies may be

28   given in amounts of 1ng/kg- 500mg/kg of patient

29   weight.  Equivalent doses of antibody fragments

30   should be used at the same or more frequent

31   intervals in order to maintain a serum level in

1     excess of the concentration that permits saturation

2     of death receptor.

3

4     Doses of the binding members may be given at any

5     suitable dose interval e.g. daily, once, twice or

6     thrice weekly.

7

8     For example, the periods of administration of a

9     humanised antibody could be from 1 bolus injection

10    to weekly administration for up to one year in

11    combination with chemotherapeutic agents.  The

12    likely dose is upwards of 1mg/per kg/per patient.

13

14    Doses of chemotherapeutic agent will depend on the

15    factors described above but preferably are

16    administered in doses which are within the normal

17    range or, preferably, at a lower concentration than

18    the normal range, wherein the normal range is the

19    range of concentrations at which the

20    chemotherapeutic agent is usually administered in

21    the absence of other therapeutic agents.

22

23    It is anticipated that in embodiments of the

24    invention the binding members and chemotherapeutic

25    agent could be given in combination with other forms

26    of chemotherapy or indeed radiotherapy.

27

28    Indeed it is believed that the advantages of the

29    invention may also be obtained when using specific

30    binding members of the invention and radiotherapy,

31    even in the absence of chemotherapeutic agents.

32

27

1    Thus, in a tenth aspect of the invention, there is

2    provided a method of killing cancer cells comprising

3    administration of a therapeutically effective amount

4    of a) a specific binding member which binds to a

5    cell death receptor or a nucleic acid encoding said

6    binding member and (b) radiotherapy treatment.

7

8    In a eleventh aspect, the present invention provides

9    a method of treating cancer comprising

10   administration of a therapeutically effective amount

11   of a) a specific binding member which binds to a

12   cell death receptor or a nucleic acid encoding said

13   binding member and (b) radiotherapy treatment to a

14   mammal in need thereof.

15

16   The specific binding member and the radiotherapy may

17   be administered simultaneously, sequentially or

18   simultaneously.  In preferred embodiments of the

19   invention, the chemotherapeutic agent is

20   administered prior to the radiotherapy.

21

22   The invention will now be described further in the

23   following non-limiting examples. Reference is made

24   to the accompanying drawings in which:

25

26   Figure 1A illustrates Northern blot analysis of Fas

27   mRNA expression in MCF-7 cells 48 hours after

28   treatment with no drug (C) or 5μM 5-FU. Equal

29   loading was assessed by analysing β-tubulin mRNA

30   expression.

31

28

1  Figure 1B illustrates Western blot analysis of Fas
2  expression in MCF-7 cells 72 hours after treatment
3  with no drug (C), 5µM 5-FU or 25nM RTX. Equal
4  loading was assessed by analysing β-tubulin
5  expression.
6
7  Figure 1C illustrates MTT cell viability assays in
8  MCF-7 cells treated with no drug (control), CH-11
9  alone (250ng/ml), 5-FU alone (5µM), or co-treated
10 with 5-FU and CH-11. The decrease in cell viability
11 for the combined treatment was highly synergistic
12 (RI=2.40, p<0.0005).
13
14 Figure 1D illustrates MTT cell viability assays in
15 MCF-7 cells treated with no drug (control), CH-11
16 alone (250ng/ml), RTX alone (25nM), or co-treated
17 with RTX and CH-11. The decrease in cell viability
18 for the combined treatment was highly synergistic
19 (RI=2.22, p<0.0005).
20
21 Figure 1E illustrates analysis of apoptosis in 5-FU
22 and CH-11 co-treated MCF-7 cells.
23
24 Figure 1F illustrates analysis of apoptosis in RTX
25 and CH-11 co-treated MCF-7 cells. Apoptosis was
26 assessed by analysing the sub-$G_1/G_0$ fraction of
27 propidium iodide stained cells by flow cytometry.
28 For both the MTT and flow cytometric analyses the
29 cells were pre-treated with each chemotherapeutic
30 drug for 72 hours followed by CH-11 for a further 24
31 hours.
32

1    Figure 2A illustrates Western blot analysis of Fas
2    expression in HCT116p53$^{+/+}$ cells treated with a range
3    of concentrations of 5-FU for 48 hours.
4
5    Figure 2B illustrates MTT cell viability assays in
6    HCT116p53$^{+/+}$ cells treated with no drug (control),
7    CH-11 alone (250ng/ml), 5-FU alone (5µM), or co-
8    treated with 5-FU and CH-11. The decrease in cell
9    viability for the combined treatment was synergistic
10   (RI=1.92, p<0.005).
11
12   Figure 2C illustrates Western blot analysis of Fas
13   expression in HCT116p53$^{+/+}$ cells treated with a range
14   of concentrations of RTX for 48 hours.
15
16   Figure 2D illustrates MTT cell viability assays in
17   HCT116p53$^{+/+}$ cells treated with no drug (control),
18   CH-11 alone (250ng/ml), RTX alone (50nM), or co-
19   treated with RTX and CH-11. The decrease in cell
20   viability for the combined treatment was highly
21   synergistic (RI=3.44, p<0.0005).
22
23   Figure 2E illustrates Western blot analysis of Fas
24   expression in RKO cells treated with a range of
25   concentrations of 5-FU for 48 hours.
26
27   Figure 2F illustrates MTT cell viability assays in
28   RKO cells treated with no drug (control), CH-11
29   alone (250ng/ml), 5-FU alone (5µM), or co-treated
30   with 5-FU and CH-11. The decrease in cell viability
31   for the combined treatment was synergistic (RI=1.74,
32   p<0.005).

30

1

2    Figure 2G illustrates Western blot analysis of Fas

3    expression in RKO cells treated with a range of

4    concentrations of RTX for 48 hours.

5

6    Figure 2H illustrates MTT cell viability assays in

7    RKO cells treated with no drug (control), CH-11

8    alone (250ng/ml), RTX alone (5nM), or co-treated

9    with RTX and CH-11. The decrease in cell viability

10    for the combined treatment was highly synergistic

11    (RI=2.31, p<0.0005).  Equal loading of Western blots

12    was assessed by analysing $\beta$-tubulin expression. For

13    each combined treatment the cells were pre-treated

14    with chemotherapeutic drug for 72 hours followed by

15    CH-11 for a further 24 hours.

16

17    Figure 3A illustrates Western blot analysis of Fas,

18    FasL, procaspase 8 and BID expression in MCF-7 cells

19    treated with $IC_{50}$ doses of 5-FU (5μM) and RTX (25nM)

20    for 72 hours. Equal loading was assessed using a $\beta$-

21    tubulin antibody.

22

23    Figure 3B illustrates Western blot analysis of Fas,

24    procaspase 8 and BID expression in MCF-7 cells

25    treated no drug (control), CH-11 alone (250ng/ml),

26    5-FU alone (5μM) for 96 hours, or co-treated with 5-

27    FU for 72 hours followed by CH-11 for a further 24

28    hours. Co-treatment with 5-FU and CH-11 resulted in

29    activation of caspase 8 and BID as indicated by

30    processing of procaspase 8 and full-length BID (lane

31    4).

32

1    Figure 3C illustrates Western blot analysis of

2    procaspase 8 and PARP expression in HCT116p53$^{+/+}$

3    cells treated with no drug (control), 5µM 5-FU or

4    50nM RTX alone or in combination with 250ng/ml CH-

5    11.

6

7    Figure 3D illustrates Western blot analysis

8    examining the kinetics of caspase 8 activation and

9    PARP cleavage in MCF-7 cells treated for 72 hours

10   with 5µM 5-FU followed by 250ng/ml CH-11 for the

11   indicated times.

12

13   Figure 3E illustrates Western blot analysing Fas,

14   procaspase 8 and PARP expression in MCF-7 cells

15   treated with 5µM 5-FU for 72 hours followed by

16   250ng/ml CH-11, 10µM IETD-fmk, or a combination of

17   CH-11 and IETD-fmk for 24 hours.

18

19   Figure 4A illustrates tetracycline (tet)-regulated

20   expression of a *TS* trans-gene in M7TS90 cells.

21

22   Figure 4B illustrates Western blot analysing the

23   effect of TS induction (-tet lanes) on Fas up-

24   regulation in M7TS90 cells in response to treatment

25   with 10µM 5-FU, 100nM RTX or 1µM MTA for 72 hours.

26                . . .

27

28

29   Figure 4C illustrates an MTT assay showing the

30   impact of TS induction (-tet) on viability of M7TS90

31   cells following treatment with 5-FU (10µM) or RTX

32

1   (100nM) in the presence of co-treatment with

2   250ng/ml CH-11.

3

4   Figure 4D illustrates the impact of TS induction on

5   caspase 8 activation and processing of full-length

6   (118kDa) PARP in M7TS90 cells treated with 5-FU

7   (10μM), RTX (100nM) or MTA (1μM) followed by

8   250ng/ml CH-11.

9

10  Figure 4E illustrates Effect of exogenous TS

11  expression on the induction of apoptosis in M7TS90

12  cells treated with 5-FU (10μM) RTX (100nM) or MTA

13  (1μM) in the presence of co-treatment with 250ng/ml

14  CH-11. Apoptosis was assessed by analysing the sub-

15  $G_1/G_0$ fraction of propidium iodide stained cells by

16  flow cytometry. Equal loading of Western blots was

17  assessed by analysing β-tubulin expression. For each

18  combined treatment the cells were pre-treated with

19  chemotherapeutic drug for 72 hours followed by CH-11

20  for a further 24 hours.

21

22  Figure 5A illustrates Western blot analysis of Fas

23  expression in p53 wild type (wt) M7TS90 and p53 null

24  (nl) M7TS90-E6 cells 72 hours after treatment with

25  no drug (Con), 10μM 5-FU, 100nM RTX or 1μM MTA.

26

27  Figure 5B illustrates MTT cell viability assays in

28  p53 null M7TS90-E6 cells treated with 10μM 5-FU,

29  100nM RTX or 1μM MTA in combination with 250ng/ml

30  CH-11.

31

1   Figure 5C illustrates Western blot analysis of

2   procaspase 8 and full-length (118kDa) PARP

3   expression in M7TS90 (wt) and M7TS90-E6 (n1) cells

4   treated with 5-FU (10µM), RTX (100nM) or MTA (1µM)

5   followed by 250ng/ml CH-11.

6

7   Figure 5D illustrates Effect of CH-11 (250ng/ml) on

8   the induction of apoptosis in M7TS90-E6 cells

9   treated with 5-FU (10µM) RTX (100nM) or MTA (1µM).

10  Apoptosis was assessed by analysing the sub-$G_1/G_0$

11  fraction of propidium iodide stained cells by flow

12  cytometry. Equal loading of Western blots was

13  assessed by analysing β-tubulin expression. For each

14  combined treatment the cells were pre-treated with

15  chemotherapeutic drug for 72 hours followed by CH-11

16  for a further 24 hours.

17

18  Figure 6A illustrates Western blot analysis of Fas

19  expression in HCT116p53$^{-/-}$ cells treated with a range

20  of concentrations of 5-FU for 48 hours.

21

22  Figure 6B illustrates MTT cell viability assays in

23  HCT116p53$^{-/-}$ cells treated with no drug (control),

24  CH-11 alone (250ng/ml), 5-FU alone (10µM), or co-

25  treated with 5-FU and CH-11. The decrease in cell

26  viability for the combined treatment was not

27  synergistic (RI=1.01).

28

29  Figure 6C illustrates Western blot analysis of Fas

30  expression in HCT116p53$^{-/-}$ cells treated with a range

31  of concentrations of RTX for 48 hours.

32

34

1    Figure 6D illustrates MTT cell viability assays in
2    HCT116p53$^{-/-}$ cells treated with no drug (control),
3    CH-11 alone (250ng/ml), RTX alone (50nM), or co-
4    treated with RTX and CH-11. The decrease in cell
5    viability for the combined treatment was synergistic
6    (RI=1.62, p=0.01).

7

8    Figure 6E illustrates Western blot analysis of Fas
9    expression in H630 cells treated with a range of
10   concentrations of 5-FU for 48 hours.

11

12   Figure 6F illustrates MTT cell viability assays in
13   H630 cells treated with no drug (control), CH-11
14   alone (250ng/ml), 5-FU alone (10μM), or co-treated
15   with 5-FU and CH-11. The decrease in cell viability
16   for the combined treatment was not synergistic
17   (RI=0.99).

18

19   Figure 6G illustrates Western blot analysis of H630
20   cells treated with a range of concentrations of RTX
21   for 48 hours.

22

23   Figure 6H illustrates MTT cell viability assays in
24   H630 cells treated with no drug (control), CH-11
25   alone (250ng/ml), RTX alone (50nM), or co-treated
26   with 5-FU and CH-11. The decrease in cell viability
27   for the combined treatment was synergistic (RI=1.41,
28   p<0.005). Equal loading of Western blots was
29   assessed by analysing β-tubulin expression. For each
30   combined treatment the cells were pre-treated with
31   chemotherapeutic drug for 72 hours followed by CH-11
32   for a further 24 hours.

1

2 **MATERIALS AND METHODS**

3 **Cell Culture.** All cells were maintained in 5% $CO_2$ at

4 37°C. MCF-7, H630 and RKO cells were maintained in

5 DMEM with 10% dialyzed bovine calf serum

6 supplemented with 1mM sodium pyruvate, 2mM L-

7 glutamine and 50µg/ml penicillin/streptomycin (from

8 Life Technologies Inc., Paisley, Scotland). M7TS90

9 cells (6) were maintained in 'MCF-7 medium'

10 supplemented with 1µg/ml puromycin, 1µg/ml

11 tetracycline (from Sigma, Poole, Dorset, England),

12 and 100µg/ml G418 (from Life Technologies Inc).

13 M7TS90-E6 cells (6) were maintained in 'M7TS90

14 medium' supplemented with 200µg/ml hygromycin (Life

15 Technologies Inc). To induce expression of exogenous

16 TS, cells were washed three times in 1xPBS and

17 incubated in growth medium lacking tetracycline.

18 HCT116 p53^{+/+} and p53^{-/-} isogenic human colon cancer

19 cells were kindly provided by Professor Bert

20 Vogelstein (John Hopkins University, Baltimore, MD).

21 HCT116 cell lines were grown in McCoy's 5A medium

22 (GIBCO) supplemented with 10% dialysed foetal calf

23 serum, 50µg/ml penicillin-streptomycin, 2mM L-

24 glutamine and 1mM sodium pyruvate.

25

26 **Northern blot analysis.** Northern blots were

27 performed as described previously using a cDNA probe

28 complementary to the *Fas* coding region (7). Equal

29 loading was assessed using a β-tubulin cDNA probe.

30

1    **Western Blotting.** Western blots were performed as

2    previously described (6). The Fas/CD95, Bcl-2 and

3    BID (Santa Cruz Biotechnology, Santa Cruz, CA),

4    caspase 8 (Oncogene Research Products, Darmstadt,

5    Germany) and PARP (Pharmingen, BD Biosciences,

6    Oxford, England) mouse monoclonal antibodies were

7    used in conjunction with a horseradish peroxidase

8    (HRP)-conjugated sheep anti-mouse secondary antibody

9    (Amersham, Little Chalfont, Buckinghamshire,

10   England). FasL rabbit polyclonal antibody (Santa

11   Cruz Biotechnology) was used in conjunction with an

12   HRP-conjugated donkey anti-rabbit secondary antibody

13   (Amersham). TS sheep monoclonal primary antibody

14   (Rockland, Gilbertsville, PA) was used in

15   conjunction with an HRP-conjugated donkey anti-sheep

16   secondary antibody (Serotech, Oxford, England).

17   Equal loading was assessed using a $\beta$-tubulin mouse

18   monoclonal primary antibody (Sigma).

19

20   **Cell Viability Assays.** Cell viability was assessed

21   by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-

22   diphenyltetrazolium bromide, Sigma) assay (12).

23   Cells were seeded at 2,500 cells per well on 96-well

24   plates 24 hours prior to drug treatment and then

25   treated with a range of concentrations of 5-FU, RTX

26   and MTA for 72 hours, following which time the

27   agonistic Fas monoclonal antibody, CH-11 (MBL,

28   Watertown, MA), was added (10-250ng/ml) for a

29   further 24 hours. MTT (0.5mg/ml) was then added to

30   each well and the cells incubated at 37°C for a

31   further 3 hours. The culture medium was removed and

32   formazan crystals reabsorbed in 200µL DMSO. Cell

1   viability was determined by reading the absorbance

2   of each well at 570nm using a 96-well microplate

3   reader (Molecular Devices, Wokingham, England).

4

5   **Flow Cytometric Analysis.** Cells were seeded at $1 \times 10^5$

6   per well of a 6-well tissue culture plate. After 24

7   hours, 5-FU, RTX or MTA were added to the medium and

8   the cells cultured for a further 72 hours, after

9   which time 250ng/ml CH-11 was added for 24 hours.

10  DNA content of harvested cells was evaluated after

11  propidium iodide staining of cells using the EPICS

12  XL Flow Cytometer (Coulter, Miami, Fl).

13

14  **Statistical Analyses.** The nature of the interaction

15  between the chemotherapeutic drugs and CH-11 was

16  determined by calculating the R index (RI), which

17  was initially described by Kern and later modified

18  by Romaneli (13, 14). The RI is calculated as the

19  ratio of expected cell survival ($S_{exp}$, defined as the

20  product of the survival observed with drug A alone

21  and the survival observed with drug B alone) to the

22  observed cell survival ($S_{obs}$) for the combination of

23  A and B ($RI = S_{exp}/S_{obs}$). Synergism is then defined as

24  an RI of greater than unity. Romanelli et al.

25  suggest that a synergistic interaction may be of

26  pharmacological interest when RI values are around

27  2.0 (14). This method was selected because treatment

28  with CH-11 alone had little effect on cell

29  viability, which meant that other methods such as

30  the median effect principle (15) and isobologram

31  methods were not suitable (16). To further assess

32  the statistical significance of the interactions,

1     the inventors designed a univariate ANOVA analysis

2     using the SPSS software package. This was an

3     additive model based on the null hypothesis that

4     there was no interaction between the drugs.

5

6     **RESULTS**

7     **Fas is highly up-regulated in response to 5-FU and**

8     **RTX.** Using DNA microarray profiling, the inventors

9     previously identified the Fas death receptor as

10    being highly up-regulated in response to 5-FU in

11    MCF-7 cells (7). Northern blot analyses confirmed

12    that Fas mRNA was up-regulated in MCF-7 cells 48

13    hours following treatment with an $IC_{60}$ dose (5µM) of

14    5-FU (Fig. 1A). Analysis of Fas protein expression

15    in MCF-7 cells revealed that it was up-regulated by

16    ~12-fold 72 hours after treatment with 5-FU (Fig.

17    1B). Fas was also highly up-regulated (by ~7-fold )

18    in response to treatment with an $IC_{60}$ dose (25nM) of

19    RTX (Fig. 1B).

20

21    **The agonistic Fas monoclonal antibody CH-11**

22    **synergistically activates apoptosis in response to**

23    **5-FU and RTX.** To examine the role of the Fas

24    signalling pathway in mediating the response of MCF-

25    7 cells to 5-FU and RTX, the inventors used the

26    agonistic Fas monoclonal antibody CH-11. Cells were

27    treated with $IC_{60}$ doses of each drug for 72 hours,

28    after which time they were treated with 250ng/ml CH-

29    11 for a further 24 hours. Treatment with 5µM 5-FU

30    alone resulted in a ~60% reduction in cell viability

31    compared to control (Fig. 1C). Treatment with CH-11

32    alone without prior incubation with 5-FU caused a

1   modest ~6% decrease in cell viability. However,

2   treatment with 5-FU followed by CH-11 was found to

3   result in an ~84% decrease in cell viability. The

4   combined treatment had an RI value of 2.40

5   indicating that the interaction was highly

6   synergistic. This was further confirmed by ANOVA

7   analysis, which indicated that the synergistic

8   interaction between the drugs was highly

9   statistically significant (p<0.0005). Similarly,

10  treatment with 25nM RTX for 72 hours followed by CH-

11  11 for 24 hours produced a highly synergistic

12  decrease in cell viability (RI=2.22, p<0.0005, Fig.

13  1D). An IgM isotype control antibody had no effect

14  on the cell viability of drug-treated cells (data

15  not shown).

16

17  To assess the degree of apoptosis in MCF-7 cells

18  treated with 5-FU and RTX individually, or in

19  combination with CH-11, the inventors carried out

20  flow cytometry of propidium iodide stained cells and

21  analysed the sub-$G_1/G_0$ apoptotic fraction. Cells were

22  treated with either 5-FU or RTX for 72 hours

23  followed by 250ng/ml CH-11 treatment for 24 hours.

24  The inventors found that CH-11 alone had little

25  effect on apoptosis (Figs. 1E and F). Treatment with

26  5-FU alone for 96 hours resulted in a modest ~2-fold

27  induction of apoptosis in response to 5µM 5-FU (Fig.

28  1E). However, addition of CH-11 to 5-FU-treated

29  cells resulted in a dramatic increase in apoptosis,

30  with a ~12-fold induction of apoptosis following co-

31  treatment with 5µM 5-FU and CH-11. Similarly, the

32  combination of RTX with CH-11 resulted in dramatic

40

1  activation of apoptosis, with ~60% of cells in the

2  sub-$G_1/G_0$ apoptotic phase following combined

3  treatment with 25nM RTX and CH-11 compared to ~11%

4  in untreated control cells, ~16% in cells treated

5  with RTX alone and ~18% in cells treated with CH-11

6  alone (Fig. 1F). The activation of apoptosis by CH-

7  11 in 5-FU and RTX treated cultures was observed

8  across a range of concentrations of each drug (Figs.

9  1E and F), indicating that the synergistic

10  interaction between CH-11 and both drugs was due to

11  activation of apoptosis.

12

13  The inventors next examined the ability of CH-11 to

14  activate apoptosis in other cell lines. Treatment of

15  HCT116p53$^{+/+}$ colon cancer cells with 5-FU resulted in

16  potent up-regulation (>10-fold) of Fas expression

17  after 48 hours (Fig. 2A). Furthermore, treatment

18  with 5µM 5-FU followed by 250ng/ml CH-11

19  synergistically decreased cell viability in this

20  line with an RI value of 1.92 (p<0.005). Similarly,

21  RTX treatment dramatically increased Fas expression

22  after 72 hours (Fig. 2C), while treatment with RTX

23  followed by CH-11 resulted in a highly synergistic

24  decrease in cell viability (Fig. 2D, RI=3.44,

25  p<0.0005). The inventors also examined another p53

26  wild type colon cancer cell line, RKO. As was the

27  case with both MCF-7 and HCT116p53$^{+/+}$ cells, both 5-

28  FU and RTX treatment resulted in dramatic Fas up-

29  regulation 48 hours post-treatment (Figs. 3E and F).

30  Furthermore, treatment of RKO cells with 5-FU or RTX

31  followed by CH-11 synergistically decreased cell

32  viability with RI values of 1.74 (p<0.0005) and 2.31

41

1   (p<0.0005) respectively (Figs. 3F and G). These

2   results indicate that CH-11 not only activates

3   apoptosis of 5-FU- and RTX-treated MCF-7 breast

4   cancer cells, but also of HCT116p53$^{+/+}$ and RKO colon

5   cancer cells. The inventors also found that

6   treatment with the antifolate MTA up-regulated Fas

7   expression and synergistically interacted with CH-11

8   to decrease cell viability in all three cell lines

9   (data not shown).

10

11  **Effect of 5-FU and RTX on Fas signal transduction.**

12  The inventors next examined drug-induced activation

13  of the Fas signalling pathway in response to 5-FU

14  and RTX. Although Fas was highly up-regulated (>10-

15  fold) in MCF-7 cells in response to $IC_{60}$ doses of

16  either drug, FasL expression was unaffected (Fig.

17  3A). Surprisingly, neither caspase 8, nor its

18  substrate BID were activated in 5-FU- or RTX-treated

19  cells as indicated by a lack of down-regulation of

20  the levels of procaspase 8 or full-length BID (Fig.

21  3A). The inventors subsequently analysed activation

22  of the Fas pathway in MCF-7 cells following co-

23  treatment with 5-FU and CH-11. Fas, procaspase 8 and

24  BID expression levels were determined in cells

25  treated with 5μM 5-FU for 72 hours followed by

26  250ng/ml CH-11 for 24 hours and compared to cells

27  treated with 5-FU alone or CH-11 alone for the

28  appropriate time periods (Fig. 3B). Treatment with

29  CH-11 alone had no effect on Fas, procaspase 8 or

30  BID expression (Fig. 3B, lane 2). As already noted,

31  treatment with 5-FU alone resulted in dramatic up-

32  regulation of Fas, but had no effect on procaspase 8

1  or BID expression, indicating that neither molecule

2  was activated (Fig. 3B, lane 3). However, treatment

3  of MCF-7 cells with 5-FU and CH-11 resulted in a

4  dramatic activation of both caspase 8 and BID as

5  indicated by complete loss of procaspase 8 and full-

6  length BID expression in these cells (Fig. 3B, lane

7  4). Similarly, in HCT116p53$^{+/+}$ cells activation of

8  caspase 8 was only observed following co-treatment

9  with either 5-FU and CH-11 or RTX and CH-11 (Fig.

10  3C). Furthermore, cleavage of PARP (poly(ADP) ribose

11  polymerase), a hallmark of apoptosis, was only

12  observed in HCT116p53$^{+/+}$ cells co-treated with each

13  drug and CH-11.

14

15  The inventors next compared the kinetics of caspase

16  8 activation with cleavage of PARP. Six hours after

17  addition of CH-11 to MCF-7 cells pre-treated for 72

18  hours with 5μM 5-FU, procaspase 8 levels were

19  reduced by ~3-fold compared to time zero (Fig. 3D).

20  This coincided with PARP cleavage, which is

21  indicative of cells undergoing apoptosis. Thus,

22  activation of caspase 8 coincided with the onset of

23  apoptosis. Twelve and 18 hours following CH-11

24  treatment, the levels of procaspase 8 had fallen to

25  less than 5% of that observed at time zero,

26  indicating potent activation of caspase 8. The

27  inventors further examined the relationship between

28  caspase 8 activation and apoptosis using the

29  specific caspase 8 inhibitor IETD-fmk. Cells were

30  pre-treated with 5μM 5-FU for 72 hours followed by

31  250ng/ml CH-11, 10μM IETD-fmk, or a combination of

32  CH-11 and IETD-fmk for 24 hours. Fas was highly up-

43

1     regulated in all treatment groups (Fig. 3D). As

2     noted above, the combination of 5-FU and CH-11

3     resulted in a dramatic activation of caspase 8 and

4     PARP cleavage (Fig. 3E, lane 2). Addition of the

5     caspase 8 inhibitor had no effect on protein

6     expression in cells treated with 5-FU alone (Fig.

7     3E, lane 3). However, IETD-fmk blocked processing of

8     procaspase 8 in cells co-treated with 5-FU and CH-11

9     (Fig. 3E, lane 4). This result indicates that

10    caspase 8 activity is necessary for procaspase 8

11    processing at the DISC and is consistent with the

12    induced proximity model proposed for caspase 8

13    activation (17). Significantly, blocking caspase 8

14    activation also inhibited PARP cleavage in 5-FU/CH-

15    11 co-treated cells, indicating that apoptosis of

16    these cells is dependent on caspase 8 activation.

17

18    **Effect of TS induction on the synergy between CH-11**

19    **and 5-FU, RTX and MTA.** Treatment with 5-FU and TS-

20    targeted antifolates has been shown to acutely

21    increase TS expression, most likely through

22    disruption of a negative feedback mechanism in which

23    TS binds to and inhibits translation of its own mRNA

24    (18). This constitutes a potential mechanism of

25    resistance as TS induction would facilitate recovery

26    of enzymatic activity. The inventors therefore

27    examined the effect of inducible TS expression on 5-

28    FU and antifolate-mediated up-regulation of Fas and

29    the synergistic interaction between CH-11 and each

30    drug. To do this, the inventors used the MCF-7-

31    derived M7TS90 cell line (6), in which transcription

32    of a TS trans-gene is activated following withdrawal

44

1    of tetracycline (tet) from the culture medium (Fig.

2    4A). In agreement with the inventors' previous

3    findings, TS induction in the M7TS90 cell line

4    abrogated RTX- and MTA-, but not 5-FU-mediated up-

5    regulation of Fas (Fig. 4B) (6). Furthermore,

6    induction of the *TS* trans-gene had little effect on

7    the synergistic interaction between 5-FU and CH-11

8    (Fig. 4C). However, TS induction completely

9    abolished the synergistic decrease in cell viability

10   caused by the combination of both 100nM RTX and CH-

11   11 and 1µM MTA and CH-11 (Fig. 4C).

12

13   The inventors next assessed the effect of inducible

14   TS on caspase 8 activation. The inventors found that

15   TS induction abrogated caspase 8 activation in

16   response to co-treatment with both antifolates and

17   CH-11, but had no effect on caspase 8 activation in

18   response to co-treatment with 5-FU and CH-11 (Fig.

19   4D). Similarly, TS induction abrogated processing of

20   PARP in cells co-treated with the antifolates and

21   CH-11, but not in cells co-treated with 5-FU and CH-

22   11 (Fig. 4D). The differential effects of TS

23   induction on apoptosis of 5-FU- and antifolate-

24   treated M7TS90 cells was further analysed by flow

25   cytometry by assessing of the sub-$G_0/G_1$ fraction in

26   cells co-treated with drug and CH-11. Co-treatment

27   with 5-FU and CH-11 resulted in a dramatic ~20-fold

28   induction of apoptosis in M7TS90 cells that was only

29   modestly reduced to ~17-fold when TS was induced

30   (Fig. 4E). In contrast, RTX and CH-11 co-treatment

31   resulted in a ~15-fold increase in the apoptoic

32   fraction, which was reduced to ~5-fold by TS

45

1   induction (Fig. 4E). Similarly, combined treatment

2   with MTA and CH-11 resulted in a dramatic ~26-fold

3   induction of apoptosis that was almost completely

4   abolished by inducible TS expression (Fig. 4E).

5   These results indicate that the activation of Fas-

6   mediated apoptosis in antifolate-treated cells was

7   highly dependent on TS expression levels. In

8   contrast, the 5-FU/CH-11 interaction was relatively

9   insensitive to TS induction in this cell line,

10  suggesting that non-TS-directed effects were

11  primarily responsible for 5-FU cytotoxicity in these

12  cells.

13

1     **Effect of p53 inactivation on the synergy between**

2     **CH-11 and 5-FU, RTX and MTA.** The inventors next

3     examined the role of p53 in the observed synergy

4     between CH-11 and each drug. p53 has been reported

5     to be an important regulator of Fas expression, both

6     transcriptionally (19) and post-transcriptionally

7     (20). The inventors previously described the

8     generation of p53 null M7TS90-E6 cells by

9     transfection of M7TS90 cells with human papilloma

10    virus (HPV)-E6 (6). Treatment of these p53 null

11    M7TS90-E6 cells with 10μM 5-FU, 100nM RTX or 1μM MTA

12    did not result in Fas up-regulation (Fig. 5A).

13    Furthermore, in contrast to the parental line, the

14    combination of 5-FU and CH-11 did not

15    synergistically decrease cell viability (RI=0.97,

16    Fig. 5B). Similarly, inactivation of p53 also

17    abolished the synergy between RTX and CH-11 and

18    between MTA and CH-11 (RI=0.85 and 1.02

19    respectively, Fig. 5B).

20

21    The inventors further examined the effects of p53

22    inactivation on drug sensitivity by comparing

23    caspase 8 activation in the p53 wild type and null

24    isogenic M7TS90 lines. Activation of caspase 8 was

25    not observed in the p53 null M7TS90-E6 cells co-

26    treated with each drug and CH-11 (Fig. 5C). In

27    contrast, caspase 8 was potently activated in the

28    parental p53 wild type cell line in response to each

29    co-treatment (Fig. 5C). Inactivation of p53 also

30    completely attenuated PARP cleavage in response to

31    co-treatment with 5-FU and CH-11 (Fig. 5C). However,

32    processing of PARP was evident in p53 null cells

1   treated with both the RTX/CH-11 and MTA/CH-11

2   combinations, although to a lesser extent than in

3   the p53 wild type line (Fig. 5C). As caspase 8 was

4   not activated, this suggests that antifolate-

5   mediated PARP cleavage in the p53 null cells was not

6   due to activation of Fas-mediated apoptosis by CH-

7   11. Indeed, the inventors found that PARP was also

8   processed in the p53 null cell line in response to

9   treatment with either RTX alone or MTA alone (data

10  not shown). These results indicate that treatment

11  with the antifolates activated p53-and Fas-

12  independent apoptosis in M7TS90-E6 cells. This was

13  further confirmed by flow cytometric analysis of

14  apoptosis in the p53 null cell line. RTX (100nM) and

15  MTA (1µM) significantly induced apoptosis of M7TS90-

16  E6 cells by ~8-fold and ~6-fold respectively 96

17  hours after drug treatment (Fig. 5D). In contrast,

18  little apoptosis was observed in M7TS90-E6 cells

19  following treatment with 10µM 5-FU (Fig. 5D).

20  Importantly, CH-11 had no significant effect on

21  apoptosis induced by any of the drugs in the p53

22  null cell line.

23

24  The inventors extended their studies into the role

25  of p53 in regulating antimetabolite-induced Fas-

26  mediated apoptosis by examining the interaction

27  between these drugs and CH-11 in the p53 null

28  HCT116p53$^{-/-}$ cell line. This cell line was derived

29  from the HCT116p53$^{+/+}$ cell line by somatic knock-out

30  of both p53 alleles (21). Compared to the p53 wild

31  type cell line, there was very little Fas induction

32  in response to 5-FU (Fig. 6A) and RTX (Fig. 6C) in

48

1   the HCT116p53$^{-/-}$ cell line, with an approximate 2-3-

2   fold induction of Fas expression observed in

3   response to 10μM 5-FU and 50nM RTX. Furthermore, no

4   synergistic interaction was observed between 5-FU

5   and CH-11 in the p53 null cell line (RI=1.01, Fig.

6   6B). Interestingly, a significant synergistic

7   interaction was still observed between RTX and CH-11

8   in HCT116p53$^{-/-}$ cells (RI=1.62, p=0.01, Fig. 6D),

9   although this was significantly less synergistic

10  than the interaction observed in the p53 wild type

11  parental line (Fig. 2D, RI=3.44, p<0.0005). This

12  suggests that RTX-mediated sensitization of HCT116

13  cells to CH-11 is not wholly p53-dependent.

14

15  The role of p53 in mediating Fas-mediated apoptosis

16  was further examined in the p53 mutant H630 colon

17  cancer cell line. Similar to the p53 null cell

18  lines, Fas was expression was not significantly

19  altered in H630 cells in response to 5-FU (Fig. 6E)

20  or RTX (Fig. 6G). No synergistic decrease in cell

21  viability was observed between 5-FU and CH-11 (Fig.

22  6F, RI=0.99), however, a statistically significant

23  synergistic interaction was observed between RTX and

24  CH-11 (Fig. 6H, RI=1.64, p<0.0005). This interaction

25  was observed despite the lack of any apparent up-

26  regulation of Fas in response to this agent,

27  suggesting that Fas expression is not the sole

28  determinant of sensitivity to CH-11 in this cell

29  line.

30  The inventors have observed similar synergistic

31  interactions between anti-Fas monoclonal antibody

32  and both TDX and oxaliplatin (data not shown) in

49

1    MCF-7 and HCT116 cell line models. Fas-targeted

2    antibodies may thus be used to stimulate apoptosis

3    in chemosensitised cancer cells.

4

5

6    **DISCUSSION**

7    The inventors have found that the Fas death receptor

8    is highly up-regulated in response to 5-FU and the

9    TS-targeted antifolates RTX and MTA in MCF-7 breast

10   cancer and HCT116p53$^{+/+}$ and RKO colon cancer cells.

11   However, this was in itself not sufficient to

12   activate caspase 8. To mimic the effects of immune

13   effector cells in their *in vitro* model, the

14   inventors used the agonistic Fas monoclonal antibody

15   CH-11. The inventors found that CH-11 potently

16   activated Fas-mediated cell death in 5-FU- and

17   antifolate-treated cells. Furthermore, the

18   interaction between CH-11 and each drug was highly

19   synergistic. The inventors' results suggest that the

20   Fas signalling pathway is an important mediator not

21   only of 5-FU-induced cell death, but also of

22   antifolate-induced cell death.

23

24   The inventors found that although FasL was not

25   induced following drug treatment, it was highly

26   expressed in MCF-7 cells. Many tumour cells

27   overexpress FasL, and it has been postulated that

28   tumour FasL induces apoptosis of Fas-sensitive

29   immune effector cells, thereby inhibiting the

30   antitumor immune response. This hypothesis has been

31   supported by both *in vitro* and *in vivo* studies (24,

32   25). The strategy of overexpressing FasL requires

1    that the tumour cells develop resistance to Fas-

2    mediated apoptosis to prevent autocrine and

3    paracrine induction of tumour cell death. Fas

4    signalling may be inhibited by a Fas splice variant

5    soluble Fas (sFas), which is a secreted protein that

6    lacks the transmembrane domain of full-length Fas

7    and may inhibit binding of FasL to Fas (26).

8    Similarly, the Fas decoy receptor DcR3 is another

9    secreted protein that binds to FasL with high

10    affinity inhibiting its interaction with Fas (27).

11    Downstream of Fas ligation, c-FLIP (FLICE-inhibitory

12    protein) and FAP-1 (Fas-associated phosphatase-1)

13    can inhibit caspase 8 recruitment and activation at

14    the Fas DISC (28, 29). The lack of caspase 8

15    activation in response to treatment with 5-FU and

16    the antifolates suggests that Fas-mediated apoptosis

17    may be inhibited in MCF-7, HCT116 and RKO cancer

18    cells. However, co-treatment with CH-11 was

19    sufficient to overcome this resistance and activate

20    Fas-mediated apoptosis.

21

22    The inventors' findings raise the possibility of

23    using antimetabolite drugs in combination with anti-

24    Fas antibodies as a novel anticancer strategy.

25    Targeting Fas may be particularly useful against

26    tumour cells that overexpress FasL and Fas pathway

27    inhibitors, and which thereby evade Fas-mediated

28    elimination by immune cells. However, systemic

29    treatment with Fas antibodies or rFasL in mouse

30    models has been shown to cause severe damage to

31    liver and other organs (31). Some recent studies

32    have focussed on local administration of rFasL, or

51

1  the use of FasL-expressing vectors as gene therapy
2  to overcome systemic toxicity (31). In addition, a
3  novel agonistic Fas-targeted antibody HFE7A has been
4  developed recently that was not hepatotoxic in
5  murine models, suggesting that it may be possible to
6  develop less toxic Fas-targeted antibodies (32).
7
8  Treatment with TS inhibitors has been shown to
9  acutely induce TS expression in cell lines and
10 tumours (18, 33). Furthermore, pre-clinical and
11 clinical studies have found that TS is a key
12 determinant of sensitivity to 5-FU, with high TS
13 expression correlating with increased resistance (1,
14 34). The inventors therefore examined the effect of
15 elevated TS expression on activation of Fas-mediated
16 apoptosis in 5-FU- and antifolate-treated cells
17 using a tetracycline-regulated TS expression system
18 (M7TS90). Interestingly, the inventors found that
19 activation of apoptosis by CH-11 in response to 5-FU
20 was not affected by increased TS expression. In
21 contrast, TS induction completely abrogated the
22 synergistic interaction between both RTX and CH-11
23 and MTA and CH-11. These findings correlated with
24 Fas expression, the up-regulation of which was
25 almost completely abrogated by TS induction in RTX-
26 and MTA-treated cells, but not 5-FU-treated cells.
27 These results indicate that the primary locus of 5-
28 FU cytotoxicity in this cell line was not TS
29 inhibition. Indeed, the inventors' previous studies
30 have suggested that misincorporation of
31 fluoronucleotides into RNA was the primary cytotoxic
32 effect of 5-FU in this line (6). Thus, despite

52

1   expressing high levels of TS, certain tumours may
2   still be sensitised to Fas-mediated apoptosis by 5-
3   FU. However, high TS expression is likely to inhibit
4   Fas-mediated apoptosis in response to folate-based
5   TS inhibitors.
6
7   Several pre-clinical studies have demonstrated that
8   loss of p53 function reduces cellular sensitivity to
9   5-FU (6, 21). Furthermore, a number of clinical
10  studies have found that p53 mutations correlated
11  with resistance to 5-FU, although other studies
12  found no such association (34). The inventors
13  assessed the effect of p53 inactivation on drug-
14  induced Fas-mediated apoptosis in two p53 wild type
15  and null isogenic cell line pairs: the MCF-7-derived
16  M7TS90 and M7TS90-E6 lines, and the HCT116p53$^{+/+}$ and
17  HCT116p53$^{-/-}$ lines. p53 inactivation attenuated Fas
18  up-regulation in response to both drugs in both cell
19  lines and inhibited the activation of apoptosis by
20  CH-11 in 5-FU- and antifolate-treated cells;
21  indicating that p53 is an important determinant of
22  Fas-mediated apoptosis in response to these agents.
23  Interestingly, some synergy was still observed
24  between RTX and CH-11 in the HCT116p53$^{-/-}$ cell line,
25  although it was significantly reduced compared to
26  the p53 wild type cell line. The inventors also
27  examined activation of Fas-mediated apoptosis in
28  response to the antimetabolites in the p53 mutant
29  H630 colon cancer cell line. Similar to the
30  HCT116p53$^{-/-}$ cell line, little Fas induction was
31  observed following drug treatment and no synergy was
32  observed between 5-FU and CH-11. However, a

1   statistically significant synergistic interaction

2   was again observed between RTX and CH-11. The

3   inventors' results surprisingly suggest that RTX

4   (but not 5-FU) can sensitize at least some cancer

5   cell lines with non-functional p53 to Fas-mediated

6   apoptosis. Furthermore, this effect appears to be

7   independent of Fas up-regulation, suggesting that

8   factors other than increased Fas expression

9   contribute to the sensitisation of tumour cells to

10  Fas-mediated apoptosis in response to this agent.

11

12  The inventors' data suggest that tumours with

13  mutated p53 would be more resistant to Fas-mediated

14  apoptosis in response to antimetabolites, in

15  particular 5-FU. However, the discriminatory p53

16  mutants Pro-175 and Ala-143 have been shown to

17  transcriptionally up-regulate Fas expression (35),

18  suggesting that certain p53 mutant tumours may be

19  sensitised to Fas-mediated cell death by

20  chemotherapy.

21

22  In conclusion, the inventors have found that the

23  agonistic Fas monoclonal antibody CH-11 dramatically

24  increases the apoptotic response to 5-FU and TS-

25  targeted antifolates in MCF-7, HCT116p53$^{+/+}$ and RKO

26  cells. Induction of exogenous TS abrogated this

27  synergistic interaction for the antifolates but not

28  5-FU, however, the extent of the interaction was

29  highly p53-dependent for each drug. The inventors'

30  findings suggest that the Fas signalling pathway is

31  an important regulator of 5-FU- and antifolate-

32  mediated cell death and that targeting the Fas

54

1     pathway in conjunction with either 5-FU or

2     antifolates may have therapeutic potential.

3

4     The inventors have observed similar synergistic

5     interactions between anti-Fas monoclonal antibody

6     and both TDX (Fig.6) and oxaliplatin (data not

7     shown) in MCF-7 and HCT116 cell line models. Fas-

8     targeted antibodies may thus be used to stimulate

9     apoptosis in chemosensitised cancer cells.

10

11     All documents referred to in this specification are

12     herein incorporated by reference. Various

13     modifications and variations to the described

14     embodiments of the inventions will be apparent to

15     those skilled in the art without departing from the

16     scope and spirit of the invention. Although the

17     invention has been described in connection with

18     specific preferred embodiments, it should be

19     understood that the invention as claimed should not

20     be unduly limited to such specific embodiments.

21     Indeed, various modifications of the described modes

22     of carrying out the invention which are obvious to

23     those skilled in the art are intended to be covered

24     by the present invention.

25

26     **REFERENCES**

27     1.    Longley, D. B., Harkin, D. P., and Johnston, P.

28         G. 5-fluorouracil: mechanisms of action and

29         clinical strategies. Nat Rev Cancer, 3: 330-

30         338, 2003.

31     2.    Hughes, L. R., Stephens, T. C., Boyle, F. T.,

32         and Jackman, A. L. Raltitrexed (Tomudex™), a

1    highly polyglutamatable antifolate thymidylate

2    synthase inhibitor, p. 147-165: Humana press,

3    1999.

4    3.    Shih, C., Chen, V. J., Gossett, L. S., Gates,

5    S. B., MacKellar, W. C., Habeck, L. L.,

6    Shackelford, K. A., Mendelsohn, L. G., Soose,

7    D. J., Patel, V. F., Andis, S. L., Bewley, J.

8    R., Rayl, E. A., Moroson, B. A., Beardsley, G.

9    P., Kohler, W., Ratnam, M., and Schultz, R. M.

10    LY231514, a pyrrolo[2,3-d]pyrimidine-based

11    antifolate that inhibits multiple folate-

12    requiring enzymes. Cancer Res, 57: 1116-1123,

13    1997.

14    4.    Aherne, G. W., Hardcastle, A., Raynaud, F., and

15    Jackman, A. L. Immunoreactive dUMP and TTP

16    pools as an index of thymidylate synthase

17    inhibition; effect of tomudex (ZD1694) and a

18    nonpolyglutamated quinazoline antifolate

19    (CB30900) in L1210 mouse leukaemia cells.

20    Biochem Pharmacol, 51: 1293-1301, 1996.

21    5.    Longley, D. B., Ferguson, P. R., Boyer, J.,

22    Latif, T., Lynch, M., Maxwell, P., Harkin, D.

23    P., and Johnston, P. G. Characterization of a

24    thymidylate synthase (TS)-inducible cell line:

25    a model system for studying sensitivity to TS-

26    and non-TS-targeted chemotherapies. Clin Cancer

27    Res, 7: 3533-3539, 2001.

28    6.    Longley, D. B., Boyer, J., Allen, W. L., Latif,

29    T., Ferguson, P. R., Maxwell, P. J., McDermott,

30    U., Lynch, M., Harkin, D. P., and Johnston, P.

31    G. The role of thymidylate synthase induction

32    in modulating p53-regulated gene expression in

1   response to 5-fluorouracil and antifolates.

2   Cancer Res, *62*: 2644-2649, 2002.

3   7.   Maxwell, P. J., Longley, D. B., Latif, T.,

4        Boyer, J., Allen, W., Lynch, M., McDermott, U.,

5        Harkin, D. P., Allegra, C. J., and Johnston, P.

6        G. Identification of 5-fluorouracil-inducible

7        target genes using cDNA microarray profiling.

8        Cancer Research, *63*, 2003.

9   12.  Mosmann, T. Rapid colorimetric assay for

10       cellular growth and survival: application to

11       proliferation and cytotoxicity assays. J

12       Immunol Methods, *65*: 55-63, 1983.

13  13.  Kern, D. H., Morgan, C. R., and Hildebrand-

14       Zanki, S. U. In vitro pharmacodynamics of 1-

15       beta-D-arabinofuranosylcytosine: synergy of

16       antitumor activity with cis-

17       diamminedichloroplatinum(II). Cancer Res, *48*:

18       117-121, 1988.

19  14.  Romanelli, S., Perego, P., Pratesi, G.,

20       Carenini, N., Tortoreto, M., and Zunino, F. In

21       vitro and in vivo interaction between cisplatin

22       and topotecan in ovarian carcinoma systems.

23       Cancer Chemother Pharmacol, *41*: 385-390, 1998.

24  15.  Chou, T. C. and Talalay, P. Quantitative

25       analysis of dose-effect relationships: the

26       combined effects of multiple drugs or enzyme

27       inhibitors. Adv Enzyme Regul, *22*: 27-55, 1984.

28  16.  Kano, Y., Ohnuma, T., Okano, T., and Holland,

29       J. F. Effects of vincristine in combination

30       with methotrexate and other antitumor agents in

31       human acute lymphoblastic leukemia cells in

32       culture. Cancer Res, *48*: 351-356, 1988.

17. Salvesen, G. S. and Dixit, V. M. Caspase activation: the induced-proximity model. Proc Natl Acad Sci U S A, *96:* 10964-10967, 1999.

18. Chu, E., Koeller, D. M., Johnston, P. G., Zinn, S., and Allegra, C. J. Regulation of thymidylate synthase in human colon cancer cells treated with 5-fluorouracil and interferon-gamma. Mol Pharmacol, *43:* 527-533, 1993.

19. Muller, M., Wilder, S., Bannasch, D., Israeli, D., Lehlbach, K., Li-Weber, M., Friedman, S. L., Galle, P. R., Stremmel, W., Oren, M., and Krammer, P. H. p53 activates the CD95 (APO-1/Fas) gene in response to DNA damage by anticancer drugs. J Exp Med, *188:* 2033-2045, 1998.

20. Bennett, M., Macdonald, K., Chan, S. W., Luzio, J. P., Simari, R., and Weissberg, P. Cell surface trafficking of Fas: a rapid mechanism of p53-mediated apoptosis. Science, *282:* 290-293, 1998.

21. Bunz, F., Hwang, P. M., Torrance, C., Waldman, T., Zhang, Y., Dillehay, L., Williams, J., Lengauer, C., Kinzler, K. W., and Vogelstein, B. Disruption of p53 in human cancer cells alters the responses to therapeutic agents. J Clin Invest, *104:* 263-269, 1999.

24. O'Connell, J., Bennett, M. W., O'Sullivan, G. C., Collins, J. K., and Shanahan, F. The Fas counterattack: a molecular mechanism of tumor immune privilege. Mol Med, *3:* 294-300, 1997.

1    25.   Bennett, M. W., O'Connell, J., O'Sullivan, G.
2          C., Brady, C., Roche, D., Collins, J. K., and
3          Shanahan, F. The Fas counterattack in vivo:
4          apoptotic depletion of tumor-infiltrating
5          lymphocytes associated with Fas ligand
6          expression by human esophageal carcinoma. J
7          Immunol, *160:* 5669-5675, 1998.

8    26.   Ruberti, G., Cascino, I., Papoff, G., and
9          Eramo, A. Fas splicing variants and their
10         effect on apoptosis. Adv Exp Med Biol, *406:*
11         125-134, 1996.

12   27.   Pitti, R. M., Marsters, S. A., Lawrence, D. A.,
13         Roy, M., Kischkel, F. C., Dowd, P., Huang, A.,
14         Donahue, C. J., Sherwood, S. W., Baldwin, D.
15         T., Godowski, P. J., Wood, W. I., Gurney, A.
16         L., Hillan, K. J., Cohen, R. L., Goddard, A.
17         D., Botstein, D., and Ashkenazi, A. Genomic
18         amplification of a decoy receptor for Fas
19         ligand in lung and colon cancer. Nature, *396:*
20         699-703, 1998.

21   28.   Krueger, A., Baumann, S., Krammer, P. H., and
22         Kirchhoff, S. FLICE-inhibitory proteins:
23         regulators of death receptor-mediated
24         apoptosis. Mol Cell Biol, *21:* 8247-8254, 2001.

25   29.   Sato, T., Irie, S., Kitada, S., and Reed, J. C.
26         FAP-1: a protein tyrosine phosphatase that
27         associates with Fas. Science, *268:* 411-415,
28         1995.

29   30.   Jiang, S., Song, M. J., Shin, E. C., Lee, M.
30         O., Kim, S. J., and Park, J. H. Apoptosis in
31         human hepatoma cell lines by chemotherapeutic

1      drugs via Fas-dependent and Fas-independent

2      pathways. Hepatology, 29: 101-110, 1999.

3  31.  Timmer, T., de Vries, E. G., and de Jong, S.

4      Fas receptor-mediated apoptosis: a clinical

5      application? J Pathol, 196: 125-134, 2002.

6  32.  Ichikawa, K., Yoshida-Kato, H., Ohtsuki, M.,

7      Ohsumi, J., Yamaguchi, J., Takahashi, S., Tani,

8      Y., Watanabe, M., Shiraishi, A., Nishioka, K.,

9      Yonehara, S., and Serizawa, N. A novel murine

10     anti-human Fas mAb which mitigates

11     lymphadenopathy without hepatotoxicity. Int

12     Immunol, 12: 555-562, 2000.

13  33.  Swain, S. M., Lippman, M. E., Egan, E. F.,

14     Drake, J. C., Steinberg, S. M., and Allegra, C.

15     J. Fluorouracil and high-dose leucovorin in

16     previously treated patients with metastatic

17     breast cancer. J Clin Oncol, 7: 890-899, 1989.

18  34.  Longley, D. B., McDermott, U., and Johnston, P.

19     G. Clinical significance of prognostic and

20     predictive markers in colorectal cancer.

21     Pharmacogenomics J, 2: 209-216, 2002.

22  35.  Munsch, D., Watanabe-Fukunaga, R., Bourdon, J.

23     C., Nagata, S., May, E., Yonish-Rouach, E., and

24     Reisdorf, P. Human and mouse Fas (APO-1/CD95)

25     death receptor genes each contain a p53-

26     responsive element that is activated by p53

27     mutants unable to induce apoptosis. J Biol

28     Chem, 275: 3867-3872, 2000.

29

30

31

32

60

1    **Claims**

2

3    1.    Use of (a) a specific binding member which
4          binds to a cell death receptor or a nucleic
5          acid encoding said binding member and (b) a
6          chemotherapeutic agent in the preparation of a
7          medicament for treating cancer.

8

9    2.    The use according to claim 1 wherein the cancer
10         is one or more of colorectal, breast , ovarian,
11         cervical, gastric, lung, liver, skin and
12         myeloid (e.g. bone marrow) cancer.

13

14   3.    The use according to claim 1 or claim 2 wherein
15         the binding member is an antibody or a fragment
16         .    thereof.

17

18   4.    The use according to any one of the preceding
19         claims wherein the death receptor is FAS.

20

21   5.    The use according to any one of the preceding
22         claims wherein the binding member is the anti-
23         FAS antibody CH11.

24

25   6.    The use according to any one of the preceding
26         claims wherein the binding member comprises at
27         least one human constant region.

28

29   7.    The use according to any one of the preceding
30         claims wherein, wherein said active agent is 5-
31         Fluorouracil or an antifolate.

32

1   8.   The use according to claim 7 wherein said
2        active agent is MTA.
3

4   9.   A method of killing cancer cells comprising
5        administering a therapeutically effective
6        amount of a) a specific binding member which
7        binds to a cell death receptor or a nucleic
8        acid encoding said binding member and (b) a
9        chemotherapeutic agent.
10

11  10.  A method of treating cancer comprising
12       administration of a therapeutically effective
13       amount of a) a specific binding member which
14       binds to a cell death receptor or a nucleic
15       acid encoding said binding member and (b) a
16       chemotherapeutic agent to a mammal in need
17       thereof.
18
19

20  11.  The method according to claim 9 or claim 10
21       wherein the cancer is one or more of
22       colorectal, breast , ovarian, cervical,
23       gastric, lung, liver, skin and myeloid (e.g.
24       bone marrow) cancer.
25

26  12.  The method according to claim 9, 10 or 11
27       wherein the binding member is an antibody or a
28       fragment thereof.
29

30  13.  The method according to any one of claims 9 to
31       12 wherein the death receptor is FAS.
32

1    14.  The method according to any one of claims 9 to
2         13 wherein the binding member is the anti-FAS
3         antibody CH11.
4
5    15.  The method according to any one of claims 9 to
6         13 wherein the binding member comprises at
7         least one human constant region.
8
9    16.  The method according to any one of claims 9 to
10        15 wherein, wherein said active agent is 5-
11        Fluorouracil or an antifolate.
12
13   17.  The method according to claim 16 wherein said
14        active agent is MTA.
15
16   18.  A product comprising a) a specific binding
17        member which binds to a cell death receptor or
18        a nucleic acid encoding said binding member and
19        (b) a chemotherapeutic agent as acombined
20        preparation for the simultaneous, separate or
21        sequential use in the treatment of cancer.
22
23   19.  A pharmaceutical composition for the treatment
24        of cancer, wherein the composition comprises a)
25        a specific binding member which binds to a cell
26        death receptor or a nucleic acid encoding said
27        binding member and (b) a chemotherapeutic agent
28        and (c) a pharmaceutically acceptable
29        excipient, diluent or carrier.
30
31   20.  The product according to claim 18 or the
32        pharmaceutical composition according to claim

1      19 wherein the cancer is one or more of

2      colorectal, breast , ovarian, cervical,

3      gastric, lung, liver, skin and myeloid (e.g.

4      bone marrow) cancer.

5

6   21.   The product according to claim 18 or claim 20

7      or the pharmaceutical composition according to

8      claim 19 or claim 20 wherein the binding member

9      is an antibody or a fragment thereof.

10

11   22.   The product according to claim 18 or claim 20

12      or 21 or the pharmaceutical composition

13      according to claim 19 or claim 20 or 21 wherein

14      the death receptor is FAS.

15

16   23.   The product according to claim 18 or any one of

17      claims 20 to 22 or the pharmaceutical

18      composition according to claim 19 or or any one

19      of claims 20 to 22 wherein the binding member

20      is the anti-FAS antibody CH11.

21

22   24.   The product according to claim 18 or any one of

23      claims 20 to 23 or the pharmaceutical

24      composition according to claim 19 or or any one

25      of claims 20 to 23 wherein the binding member

26      comprises at least one human constant region.

27

28   25.   The product according to claim 18 or any one of

29      claims 20 to 24 or the pharmaceutical

30      composition according to claim 19 or or any one

31      of claims 20 to 24 wherein, wherein said active

64

1    agent is 5-Fluorouracil or an antifolate.

2

3    26.    The product or pharmaceutical composition
4           according to claim 25 wherein said active agent
5           is MTA.

6

7    27.    27.    A kit for the treatment of cancer, said
8           kit comprising:
9           a) a specific binding member which binds to a
10          cell death receptor or a nucleic acid encoding
11          said binding member and (b) a chemotherapeutic
12          agent and
13          (c) instructions for the administration of (a)
14          and (b) separately, sequentially or
15          simultaneously.

16

17

18

# 1/20

## Figure 1A

C          5-FU

Fas

β-tubulin

## Figure 1B

C        5-FU      C        RTX

Fas

β-tubulin
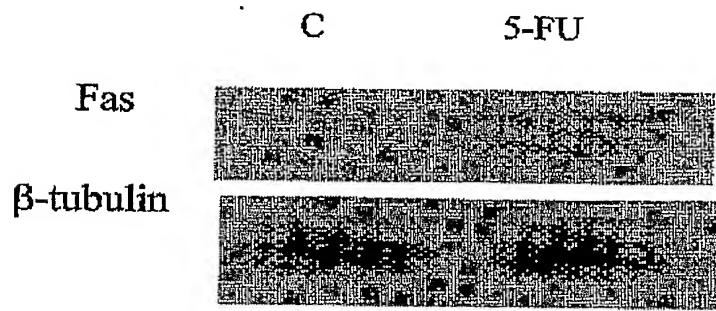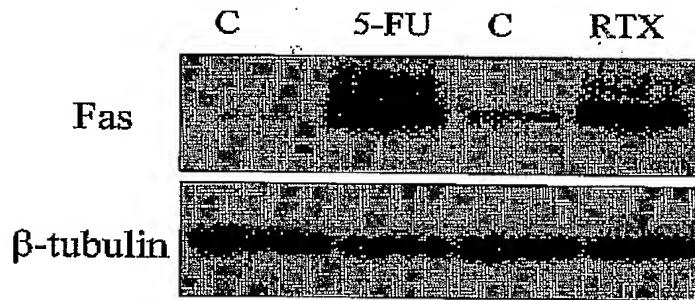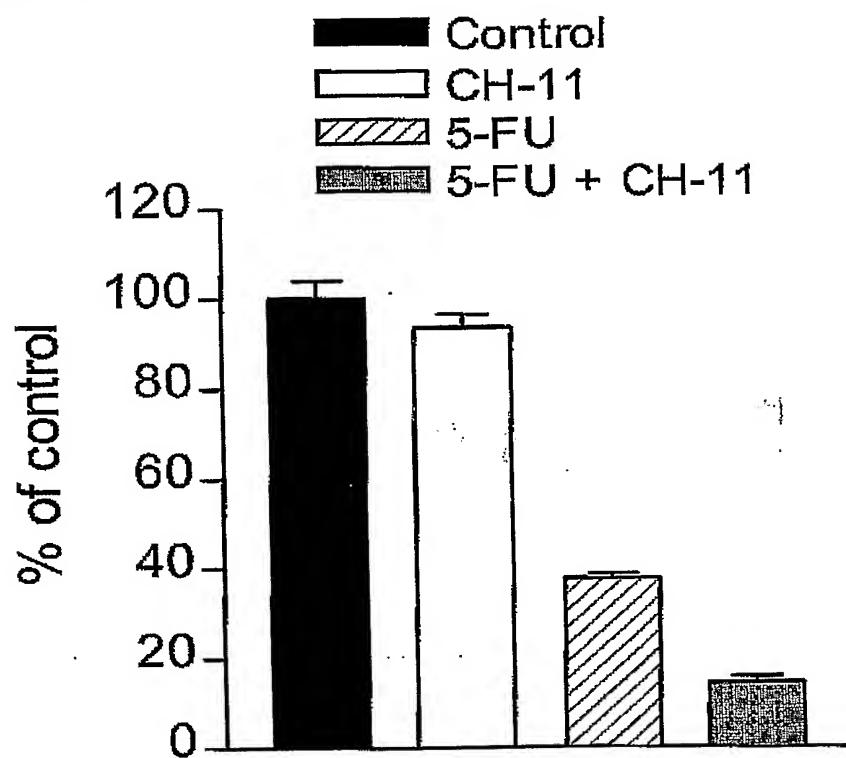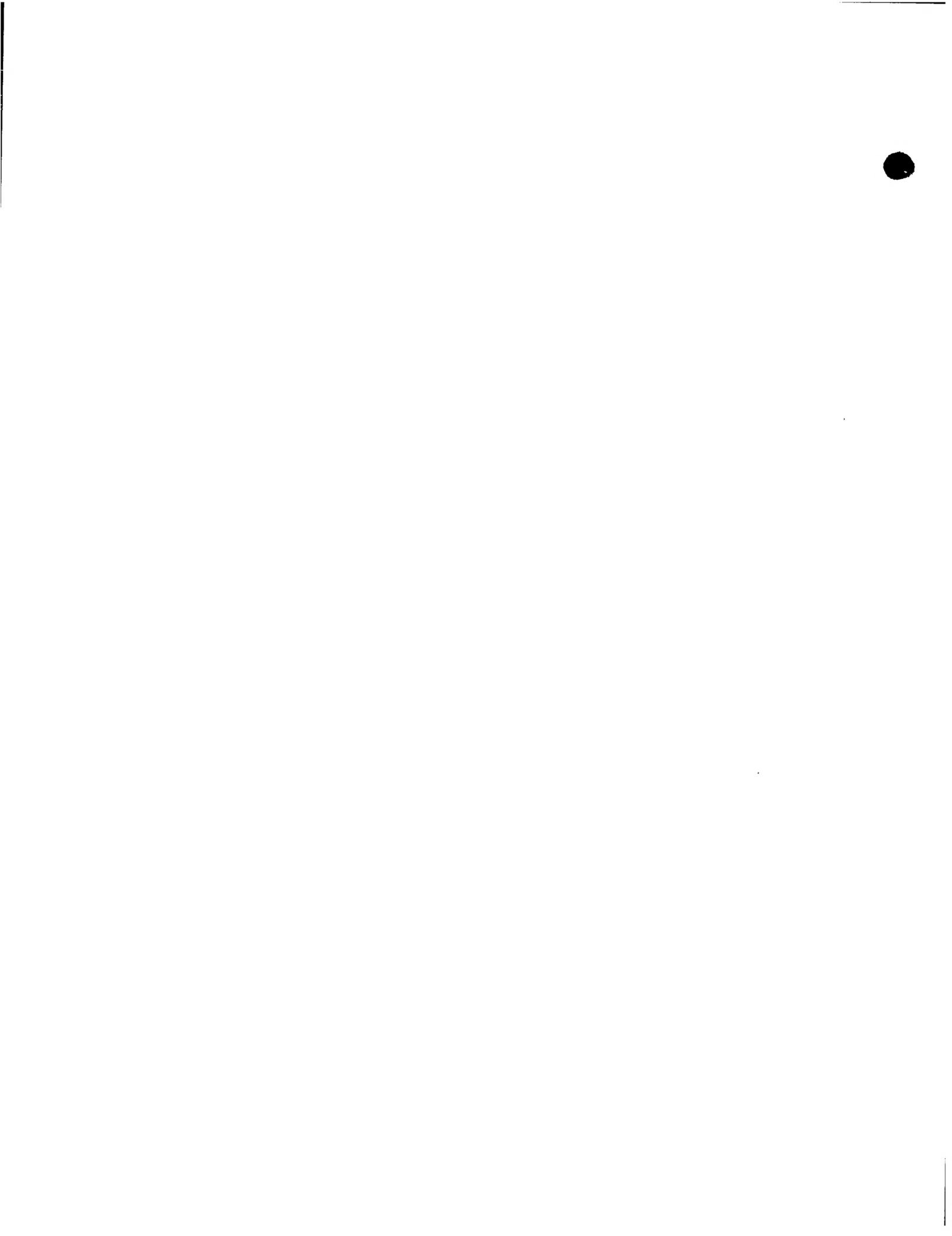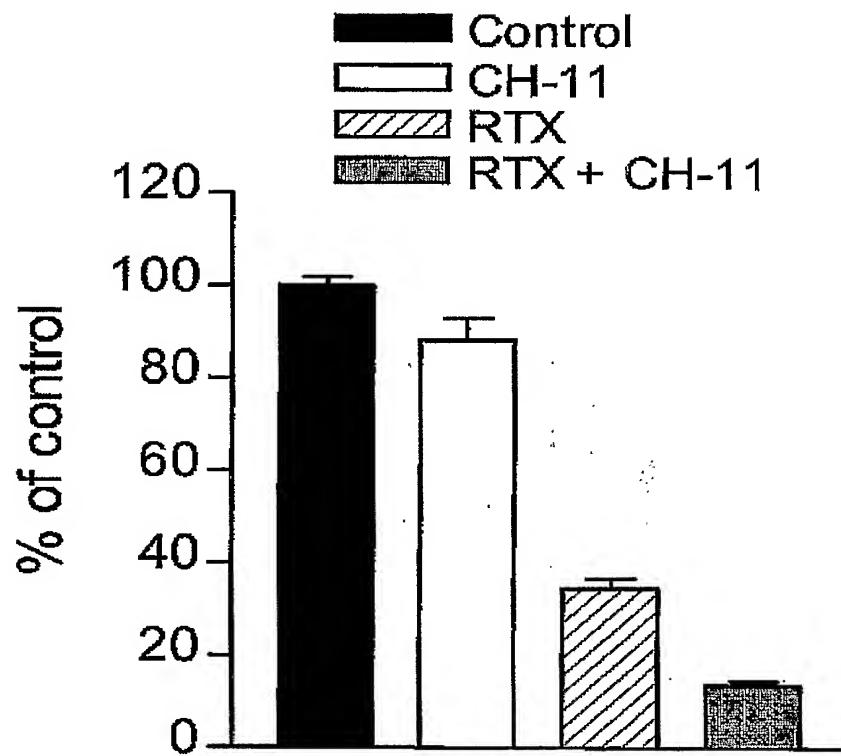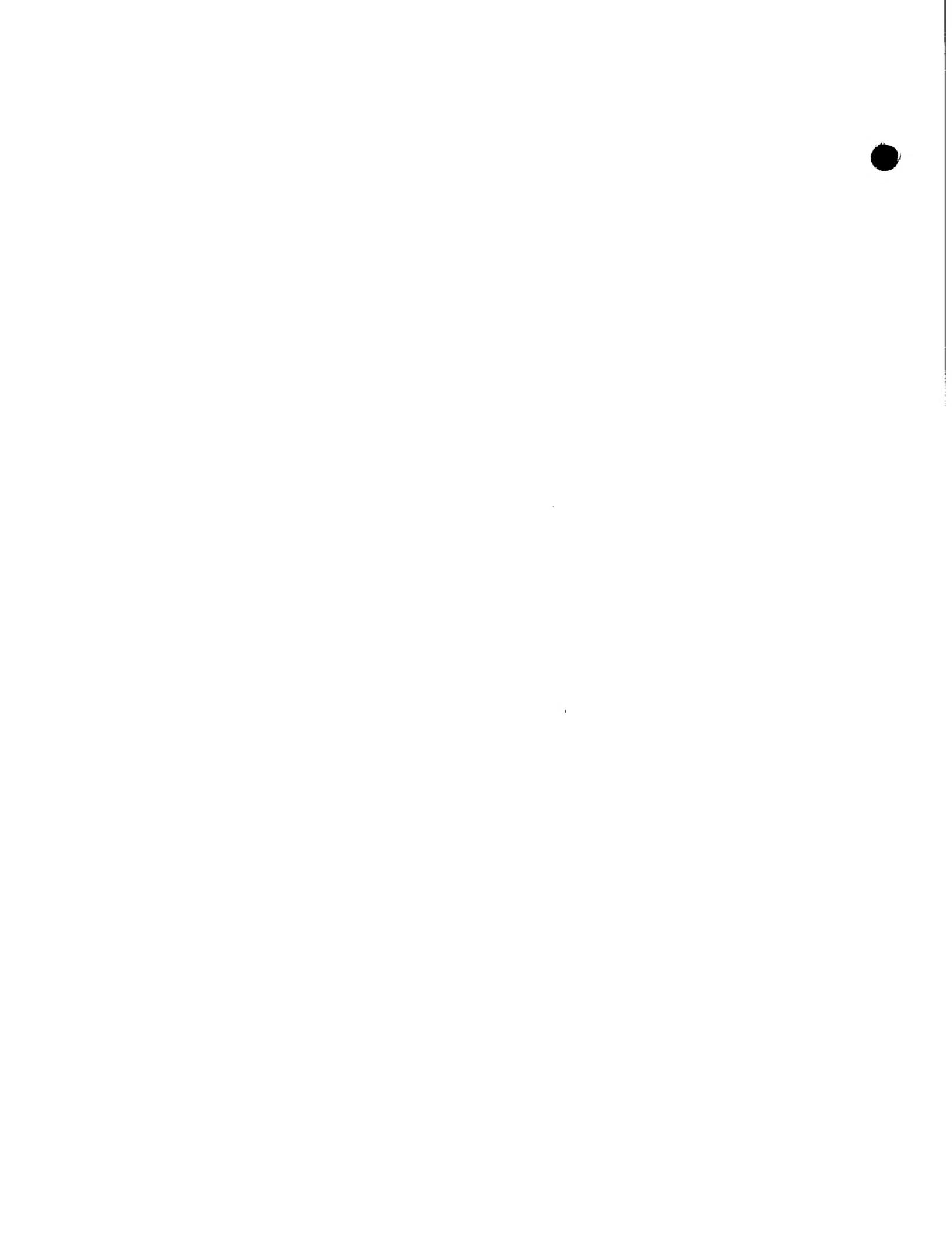
Figure 1C

Figure 1D

4/20
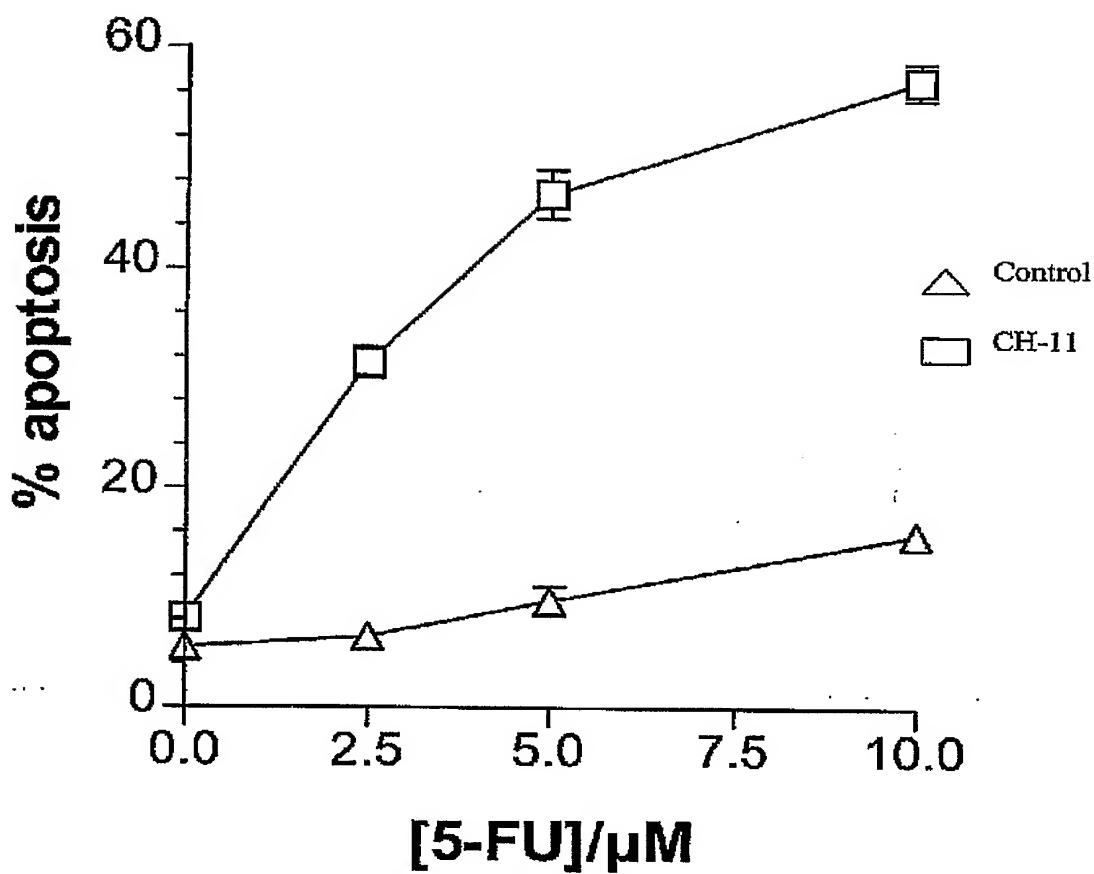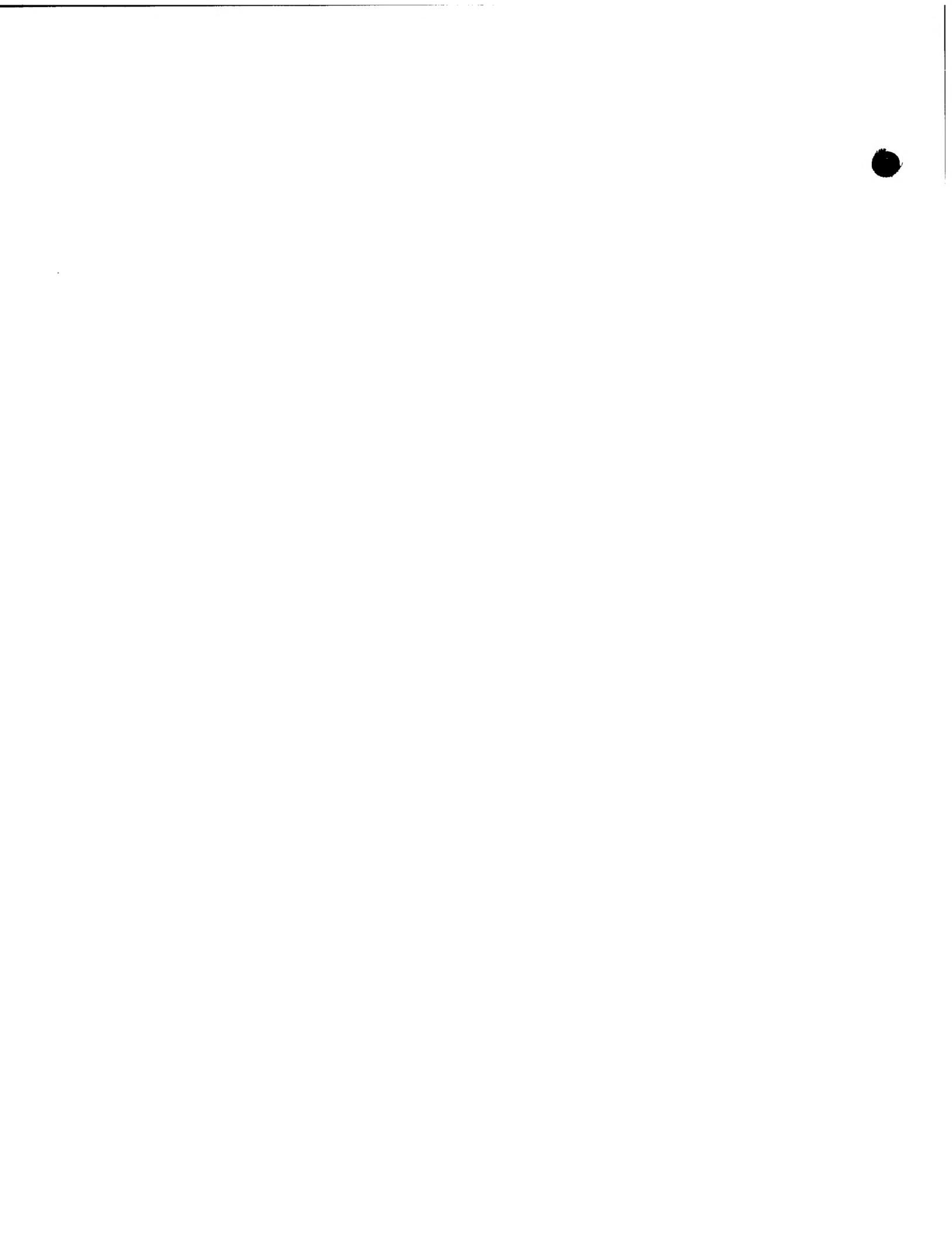


Figure 1E

5/20
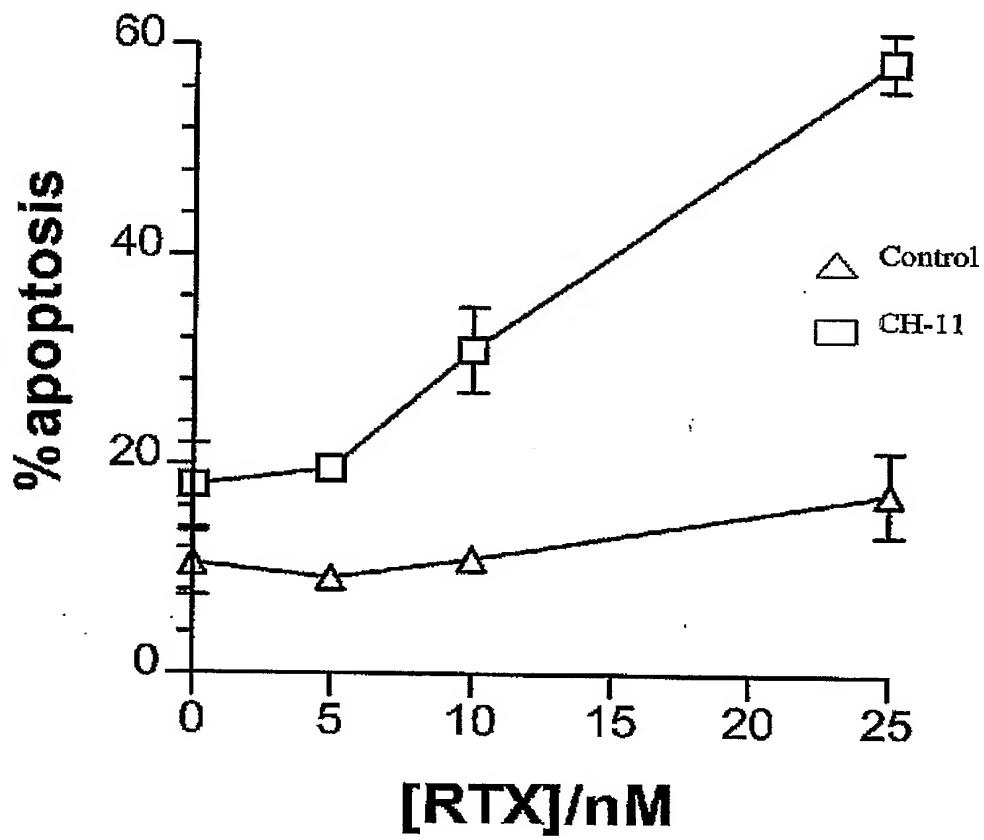


Figure 1F
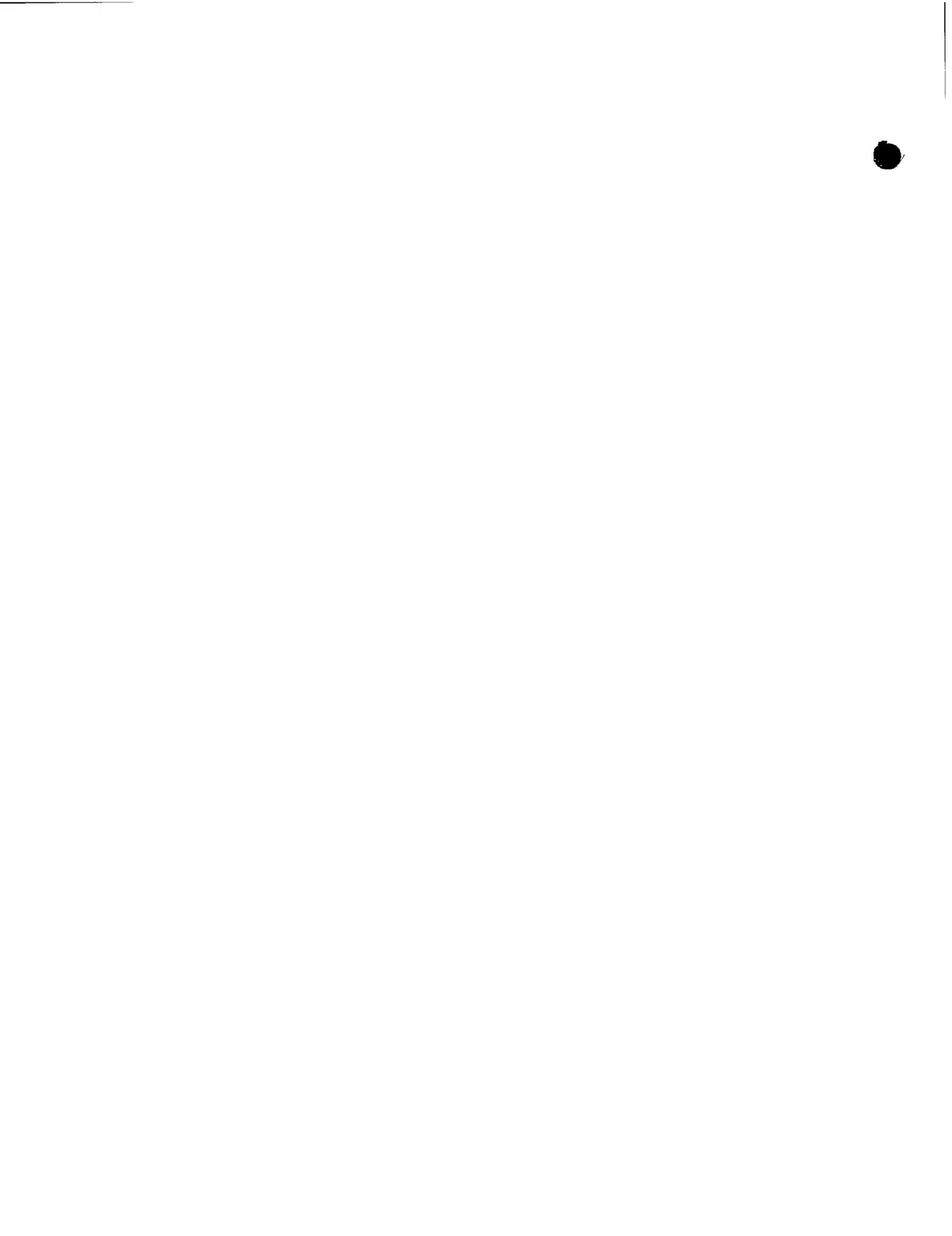
6/20

Figure 2A

HCT116p53$^{+/+}$

| | 0 | 5 | 10 | 20 | μM 5-FU |



Fas

β-tubulin



Figure 2B

7/20

HCT116p53^{+/+}

| 0 | 10 | 50 | 100 | nM RTX |

Fas

β-tubulin

Figure 2C



Figure 2D

RKO

0    5    10    μM 5-FU

Fas

β-tubulin

Figure 2E

■ Control
□ CH-11
▨ 5-FU
▩ 5-FU + CH-11

% of control

120

100

80

60

40

20

0

Figure 2F

9/20

RKO

| 0 | 10 | 100 | nM RTX |

Fas

β-tubulin
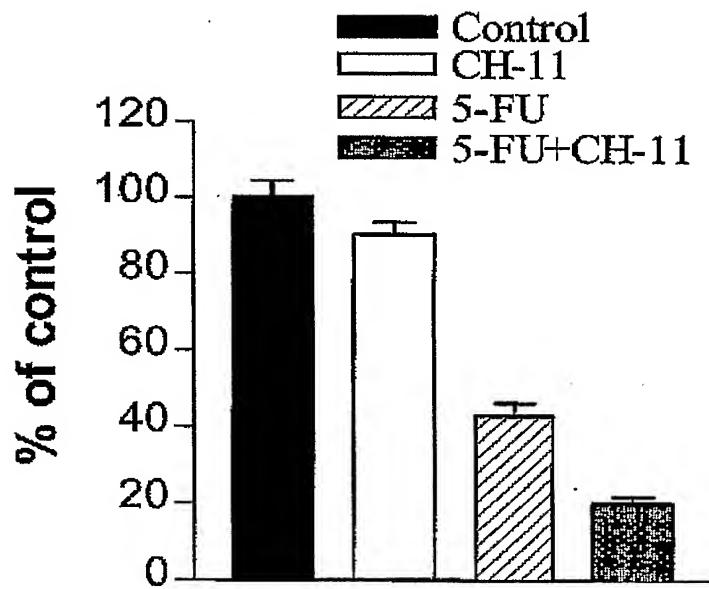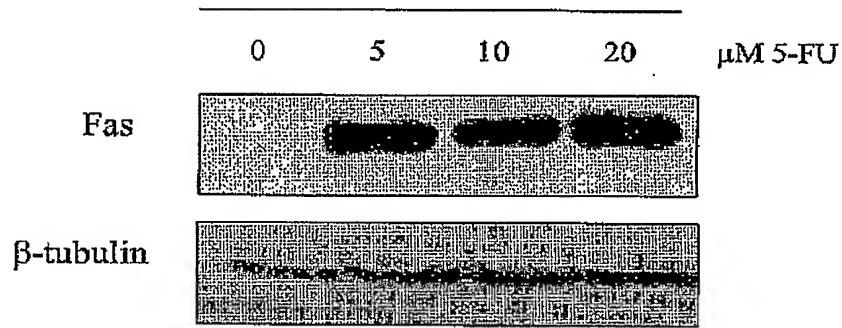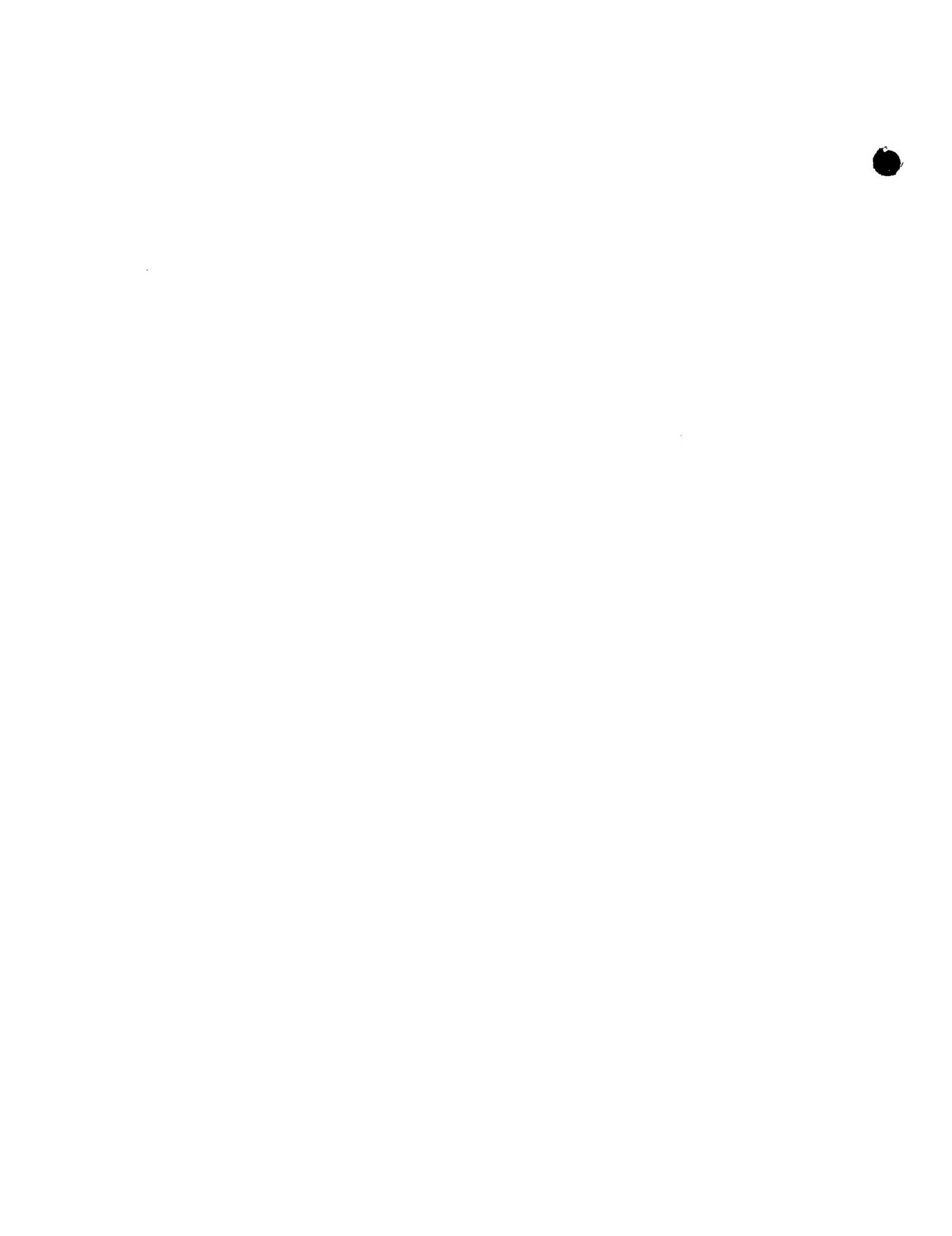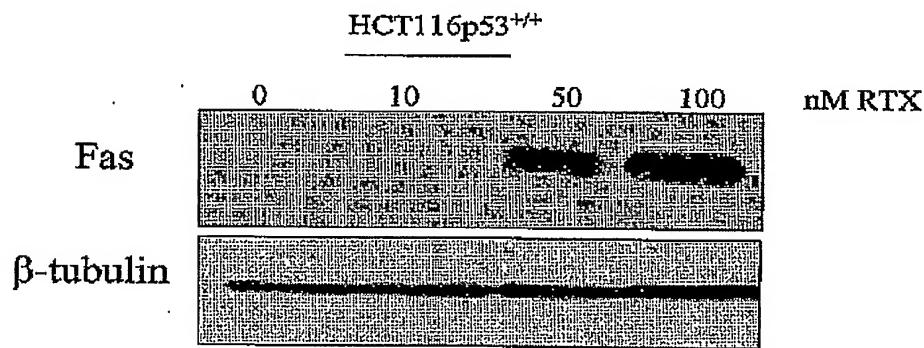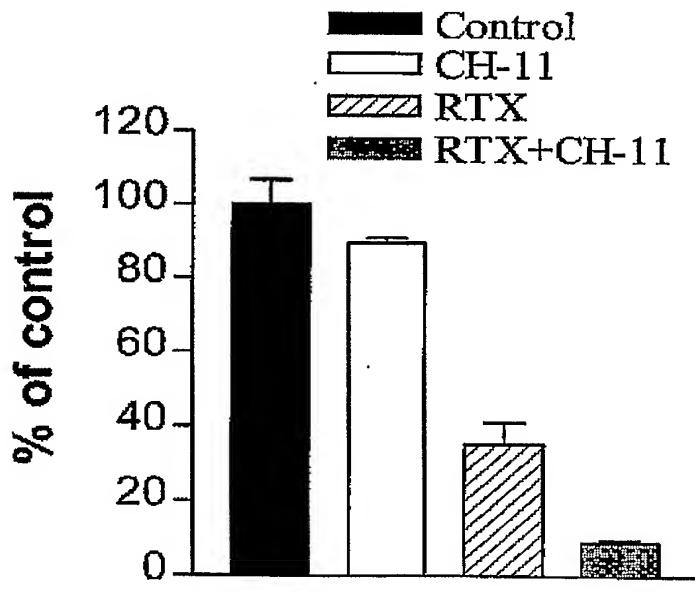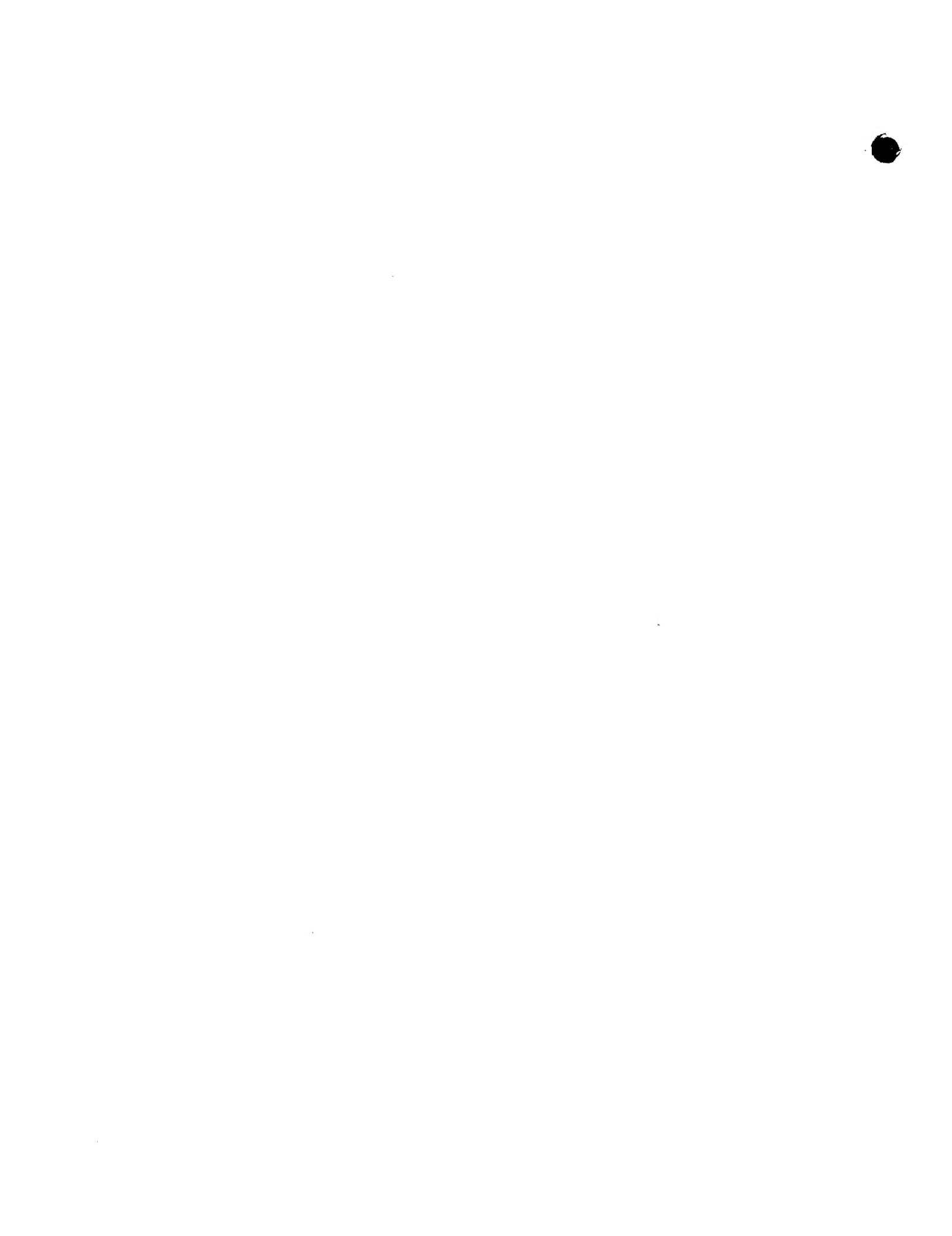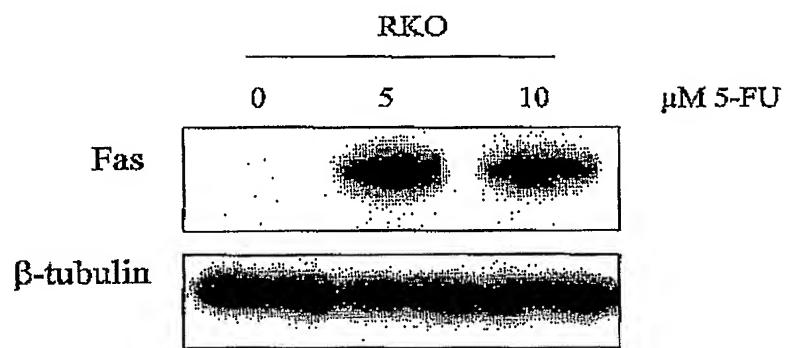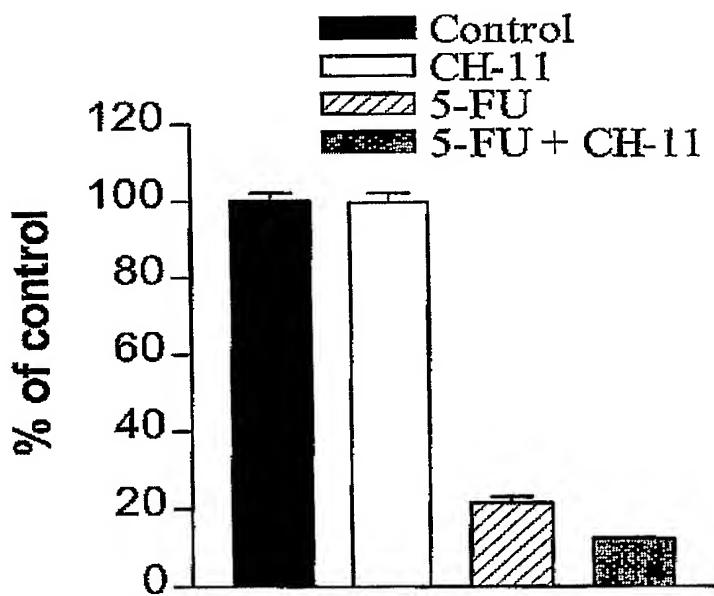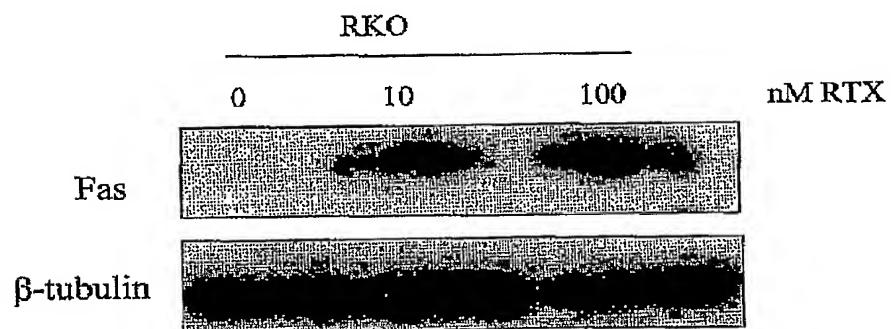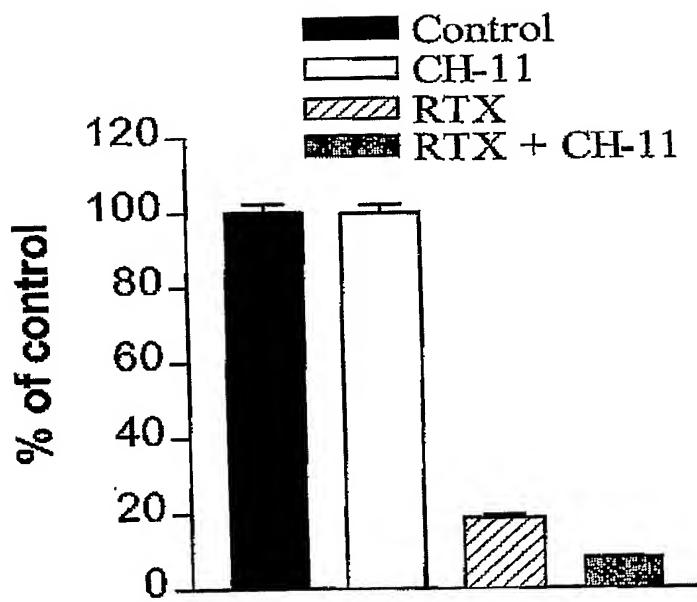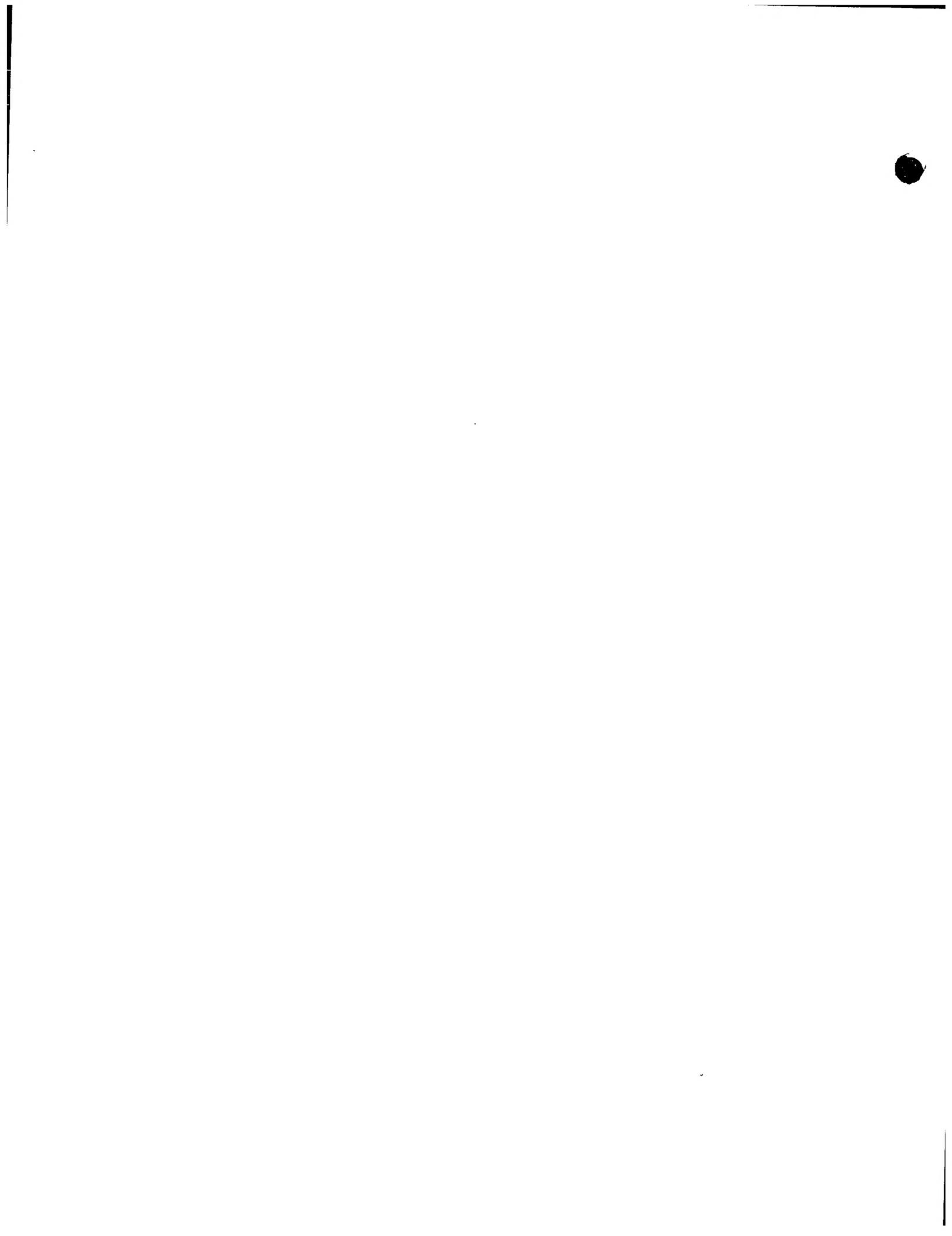
Figure 2G

Figure 2H

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Figure 3A
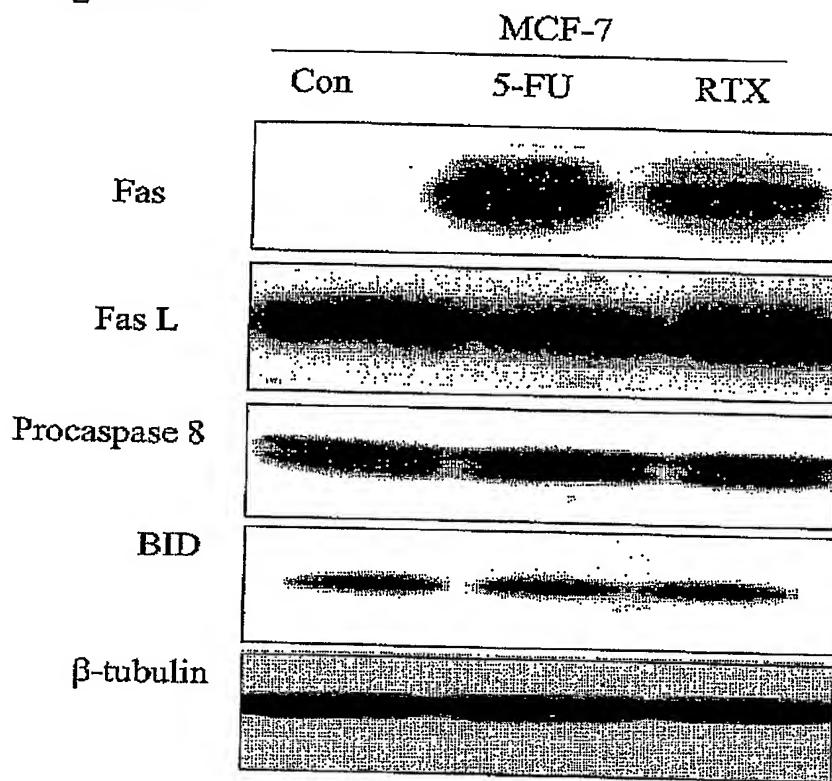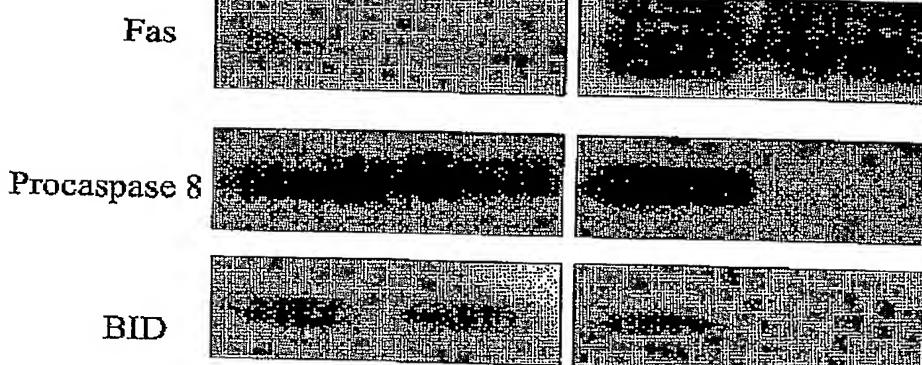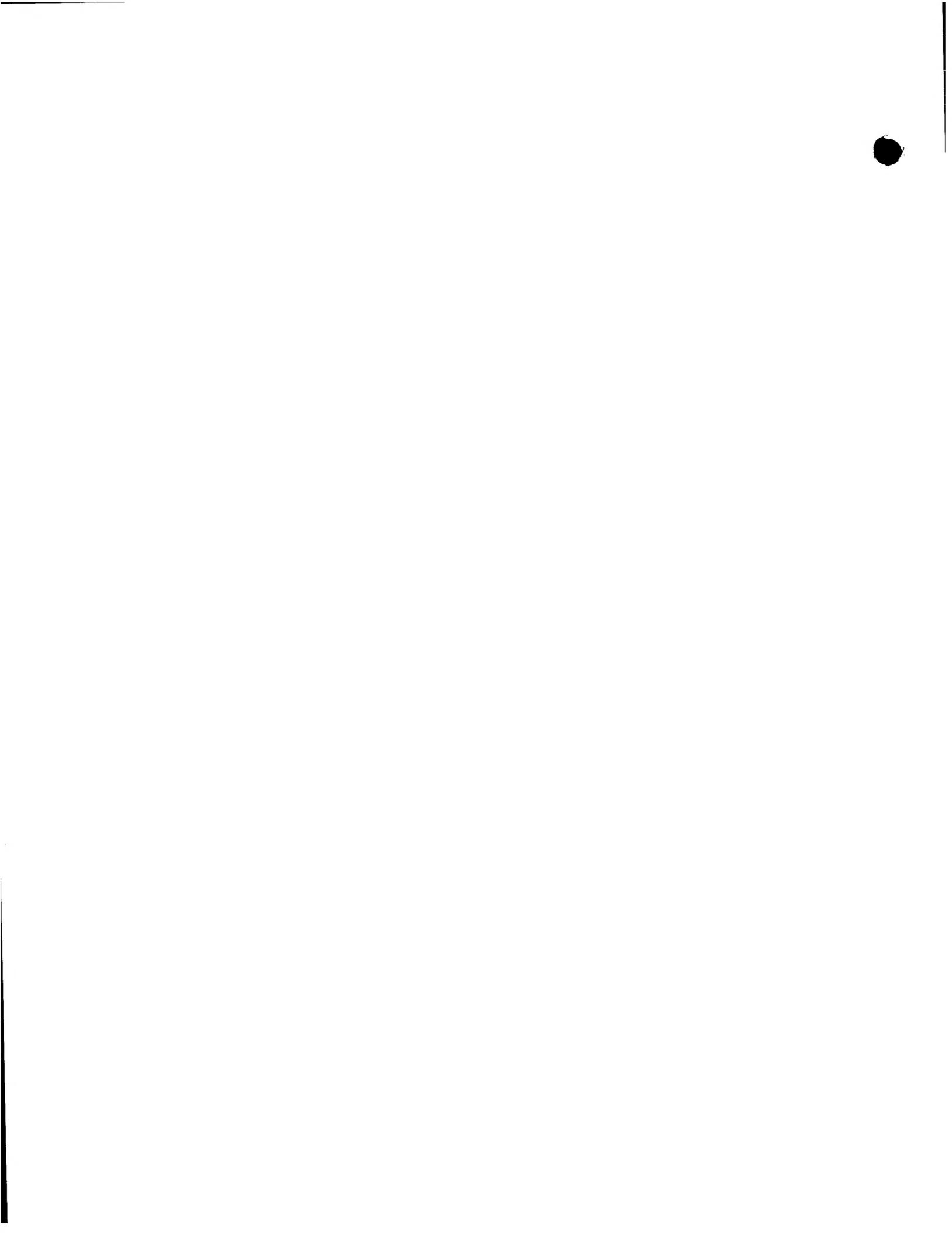


Figure 3B

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## Figure 3C

HCT116p53$^{+/+}$

| Con | 5-FU | RTX |
| --- | --- | --- |
| − + | − + | − + | −/+ CH-11 |
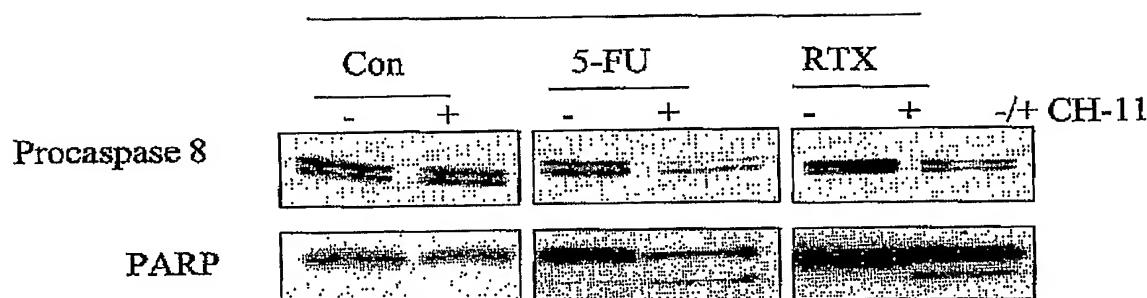
Procaspase 8

PARP

MCF-7

## Figure 3D

0      3      6      12      18    hours post-CH-11

Procaspase 8
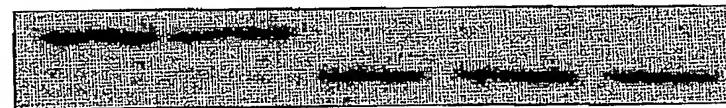
PARP

## Figure 3E

MCF-7

1   2   3   4

Fas
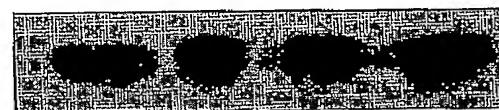
Procaspase 8

PARP

1= 5-FU
2= 5-FU+CH-11
3= 5-FU+fmk
4= 5-FU+CH-11+fmk
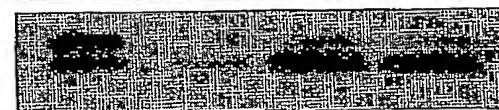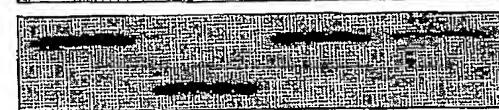
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Figure 4A    M7TS90
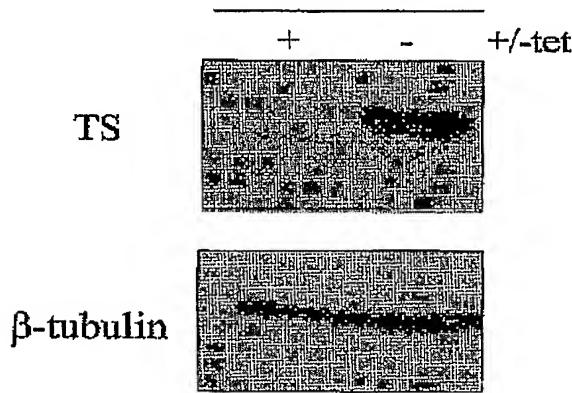


Figure 4B
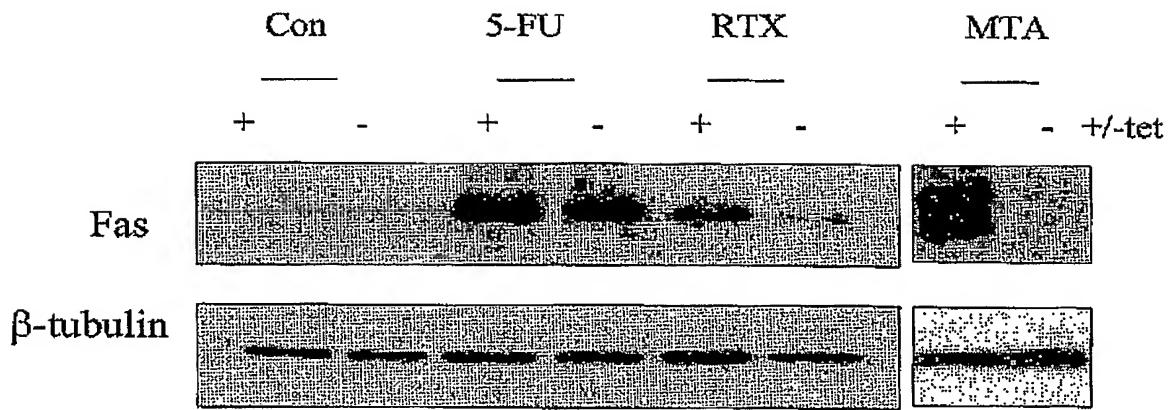
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Figure 4C
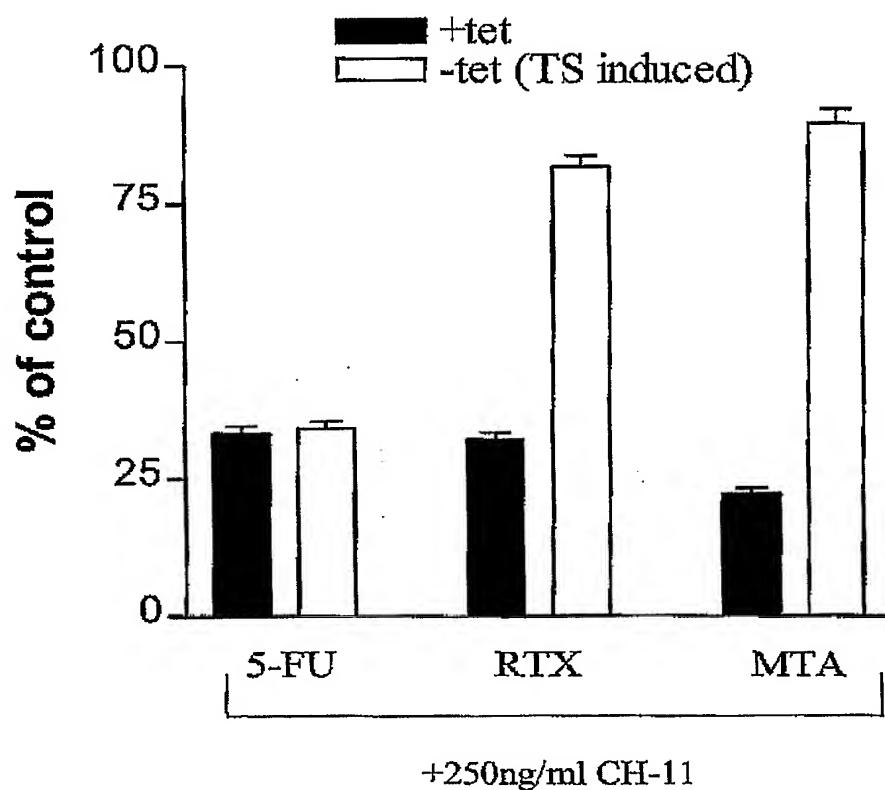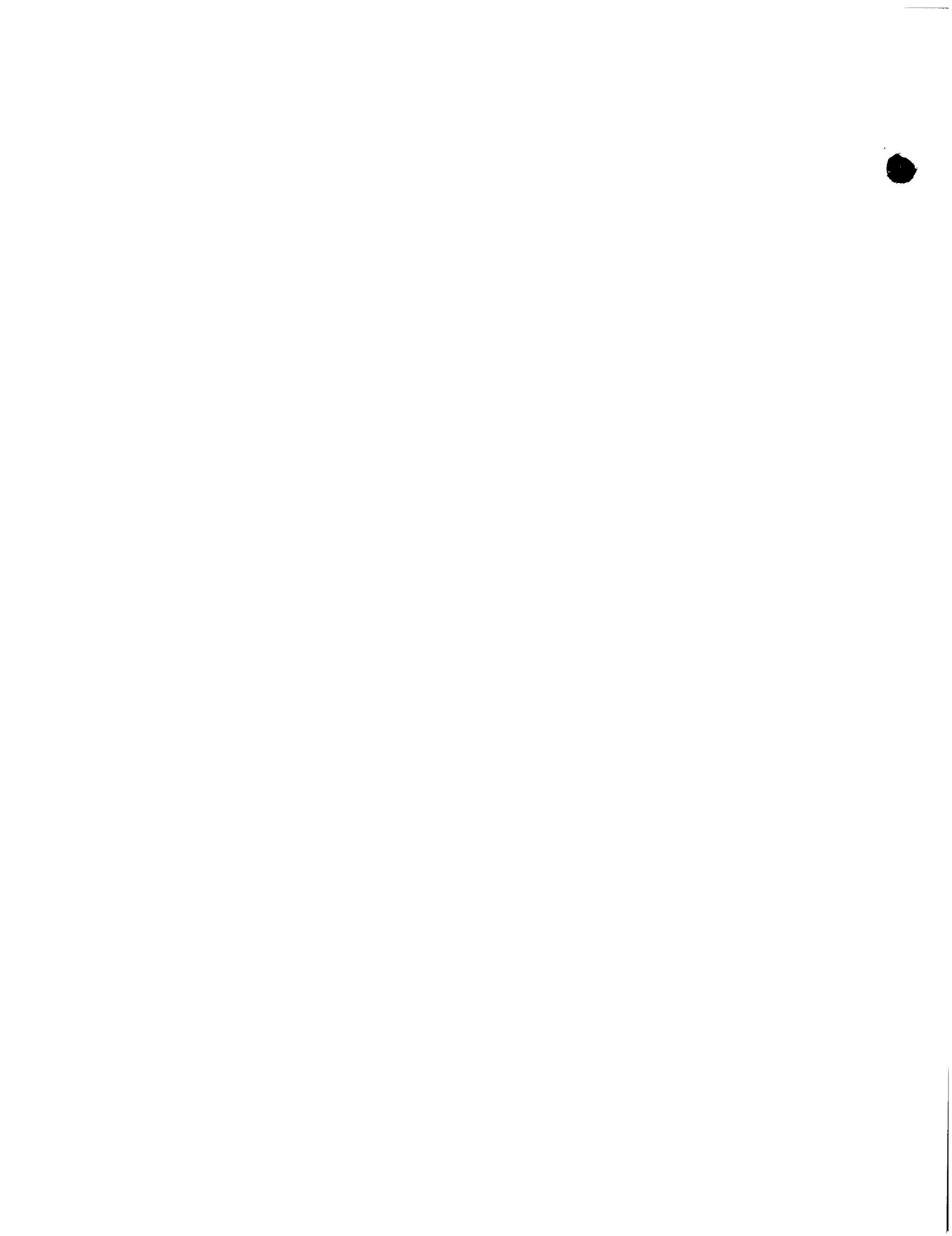


+250ng/ml CH-11
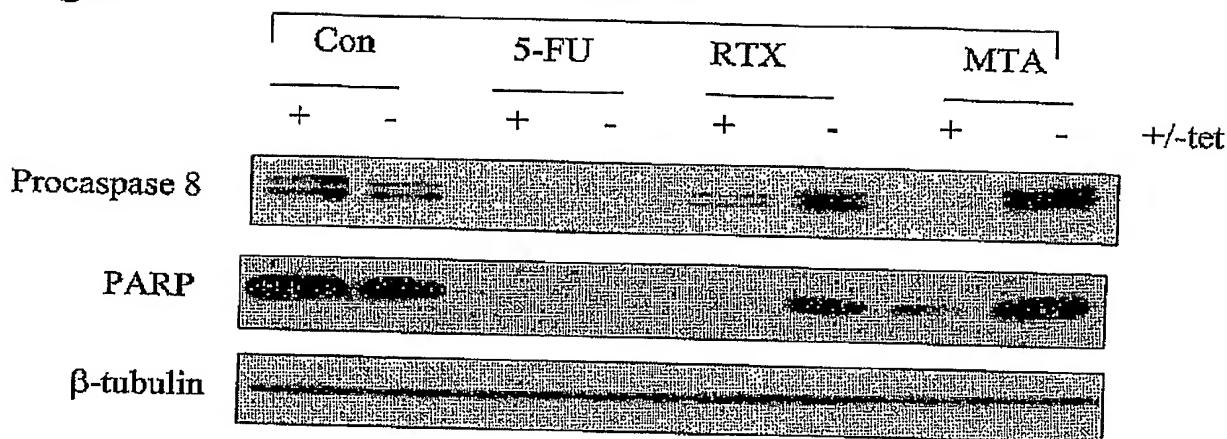
Figure 4D
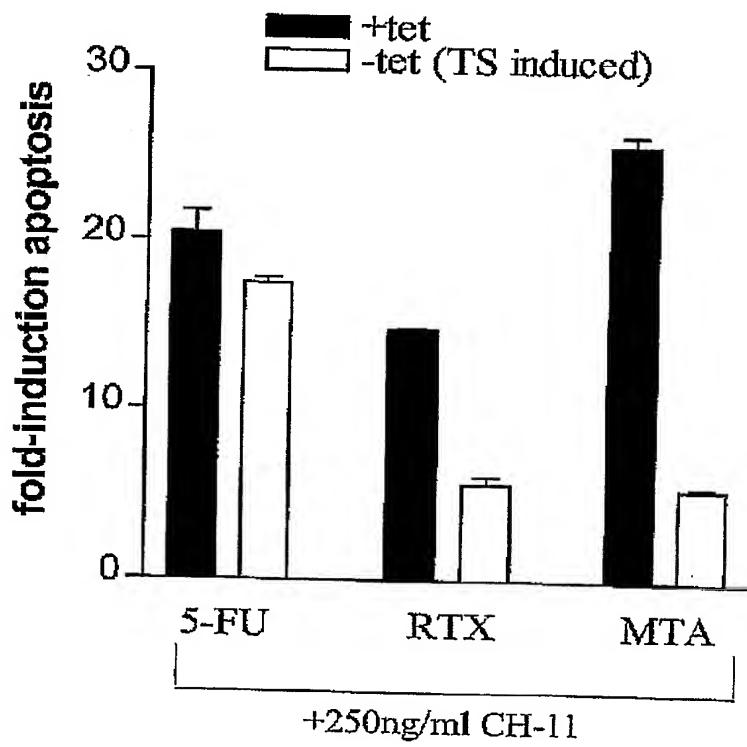


Figure 4E

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Figure 5A
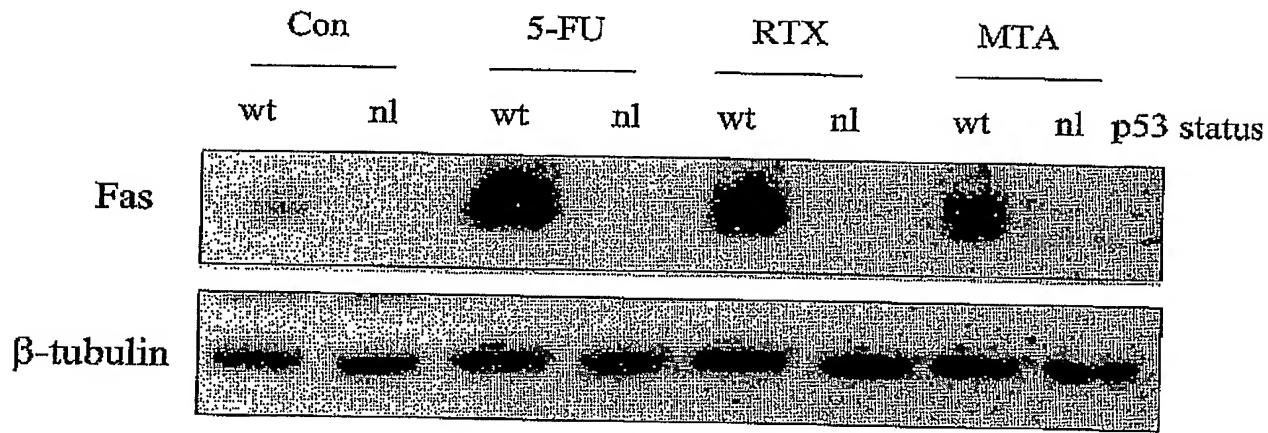


Figure 5B
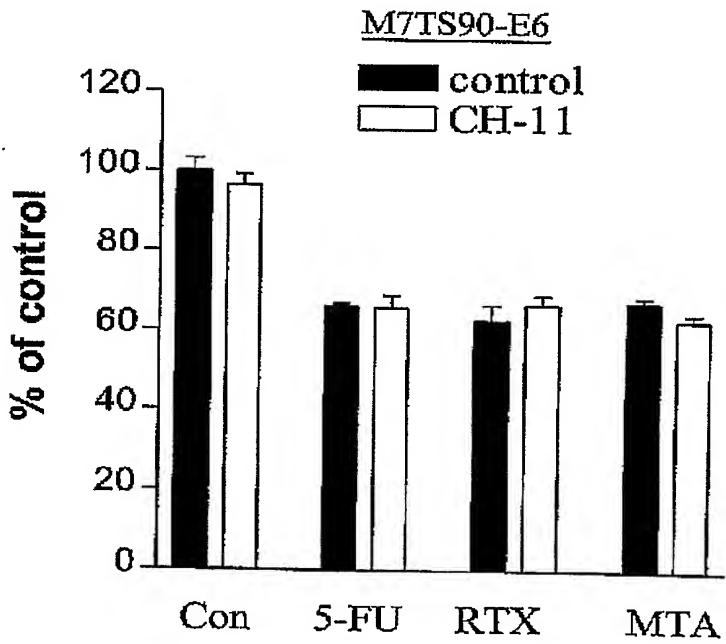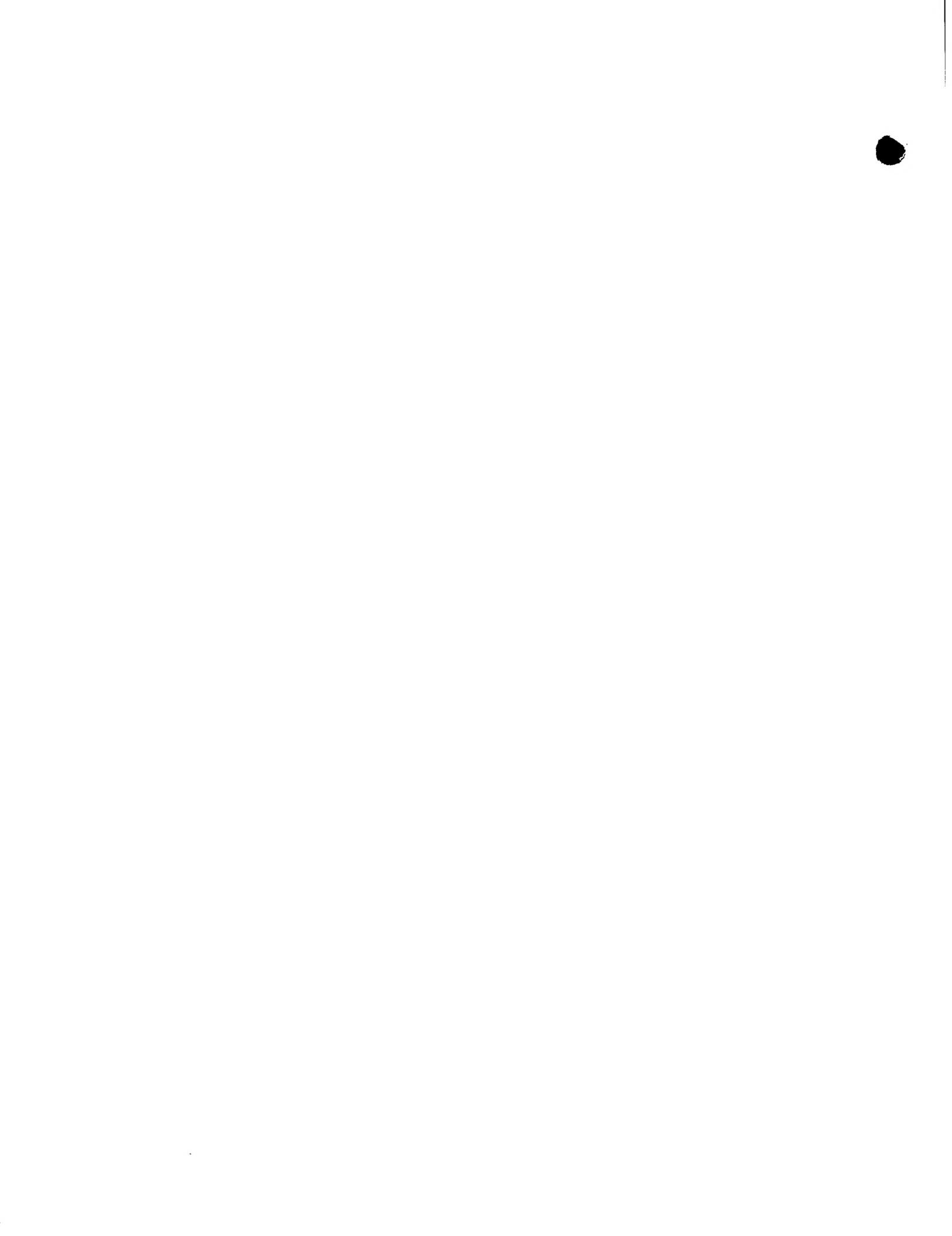
## Figure 5C

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+250ng/ml CH-11
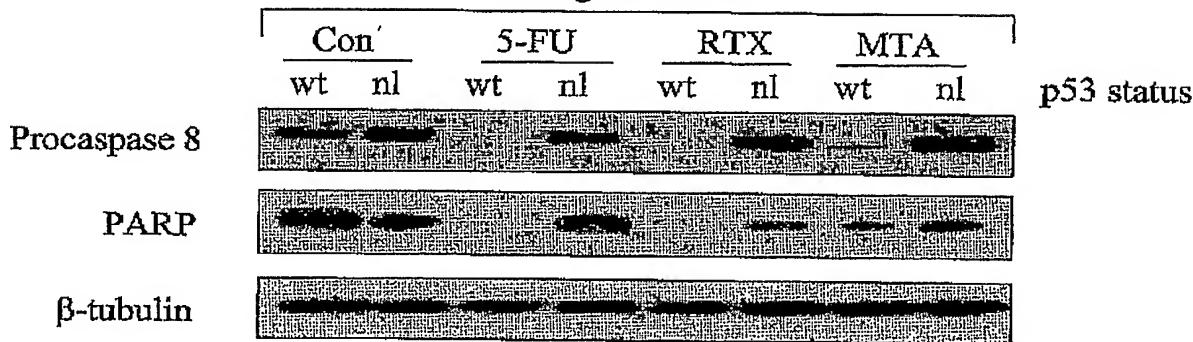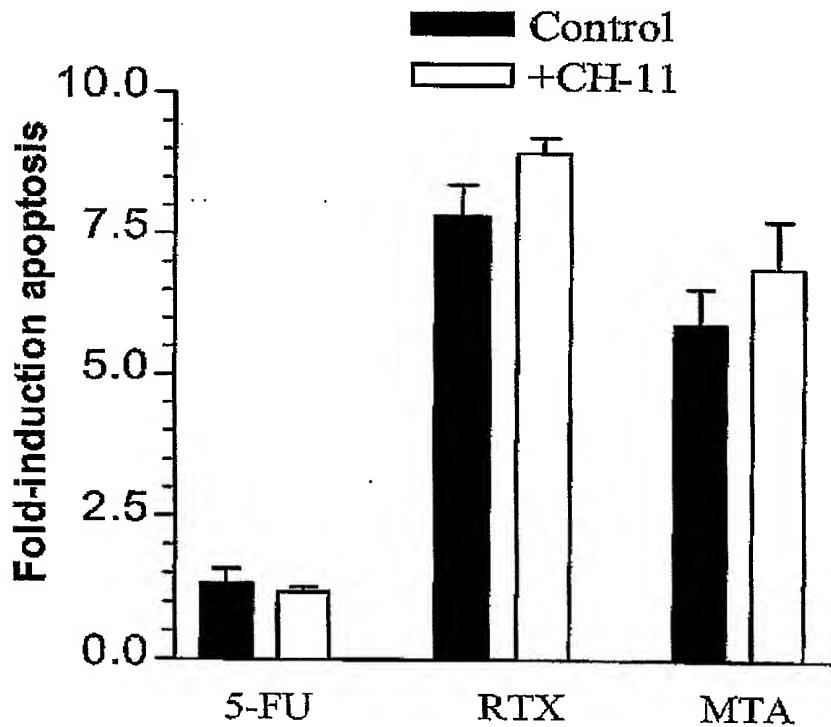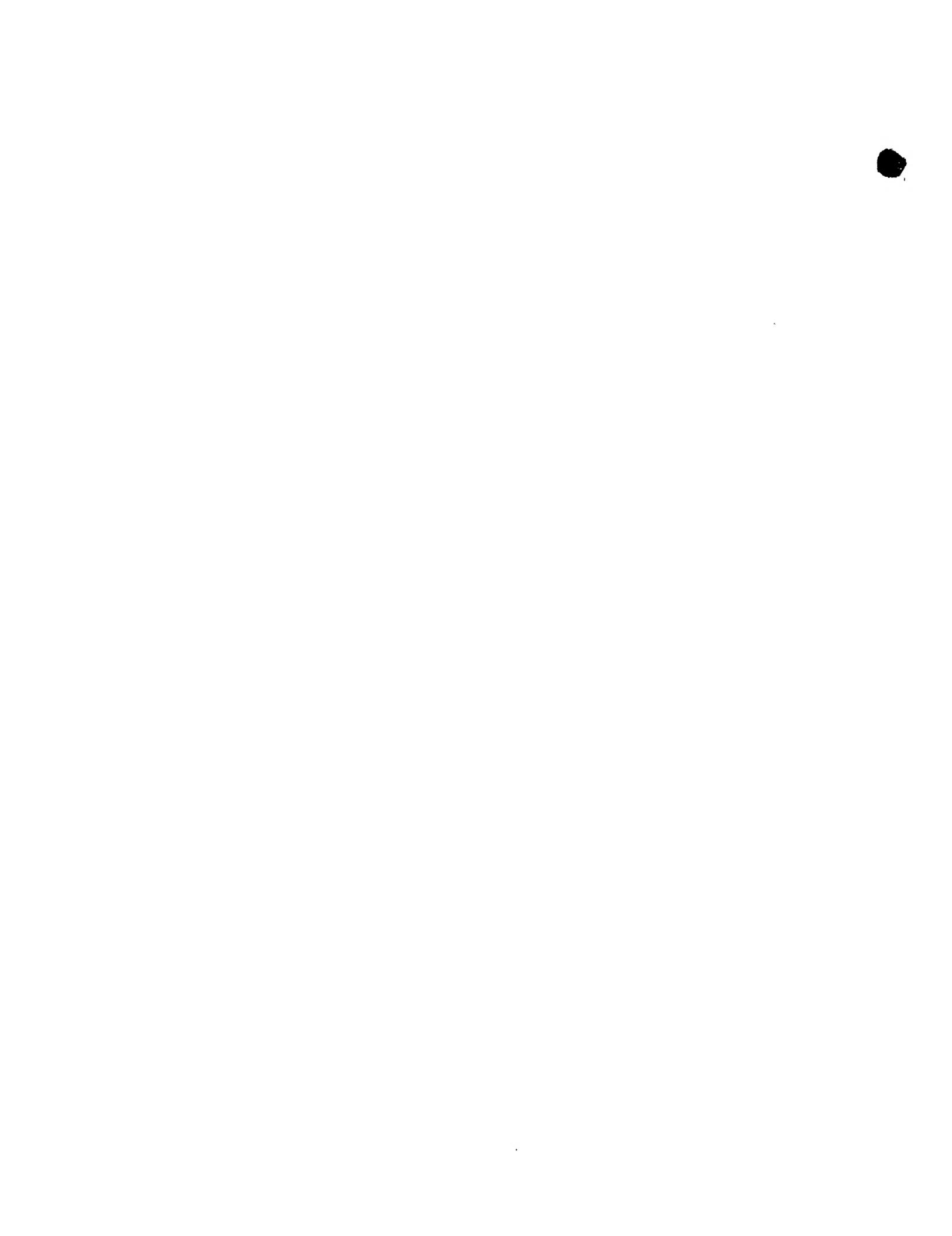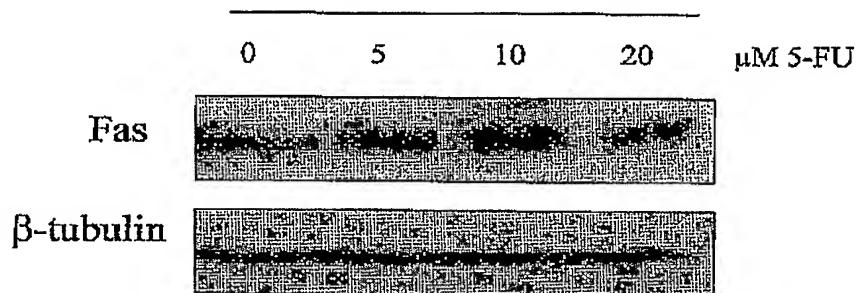
| | Con | | 5-FU | | RTX | | MTA | | |
|---|---|---|---|---|---|---|---|---|---|
| | wt | nl | wt | nl | wt | nl | wt | nl | p53 status |

Procaspase 8

PARP

β-tubulin

## Figure 5D

# M7TS90-E6

■ Control
☐ +CH-11

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Figure 6A



Figure 6B

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Figure 6C

p53$^{-/-}$

| 0 | 10 | 50 | 100 | nM RTX |

Fas

β-tubulin



Figure 6D



■ Control
□ CH-11
▨ RTX
▒ RTX + CH-11

Figure 6E

H630

| 0 | 5 | 10 | 20 | µM 5-FU |

Fas

β-tubulin

Figure 6F



Legend:
- ■ Control
- □ CH-11
- ▨ 5-FU
- ▤ 5-FU + CH-11

% of control (y-axis: 0, 20, 40, 60, 80, 100, 120)
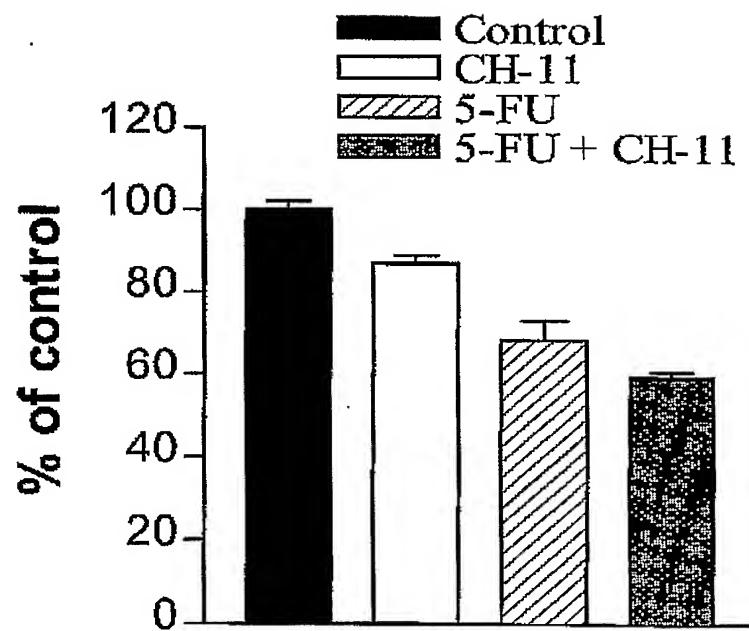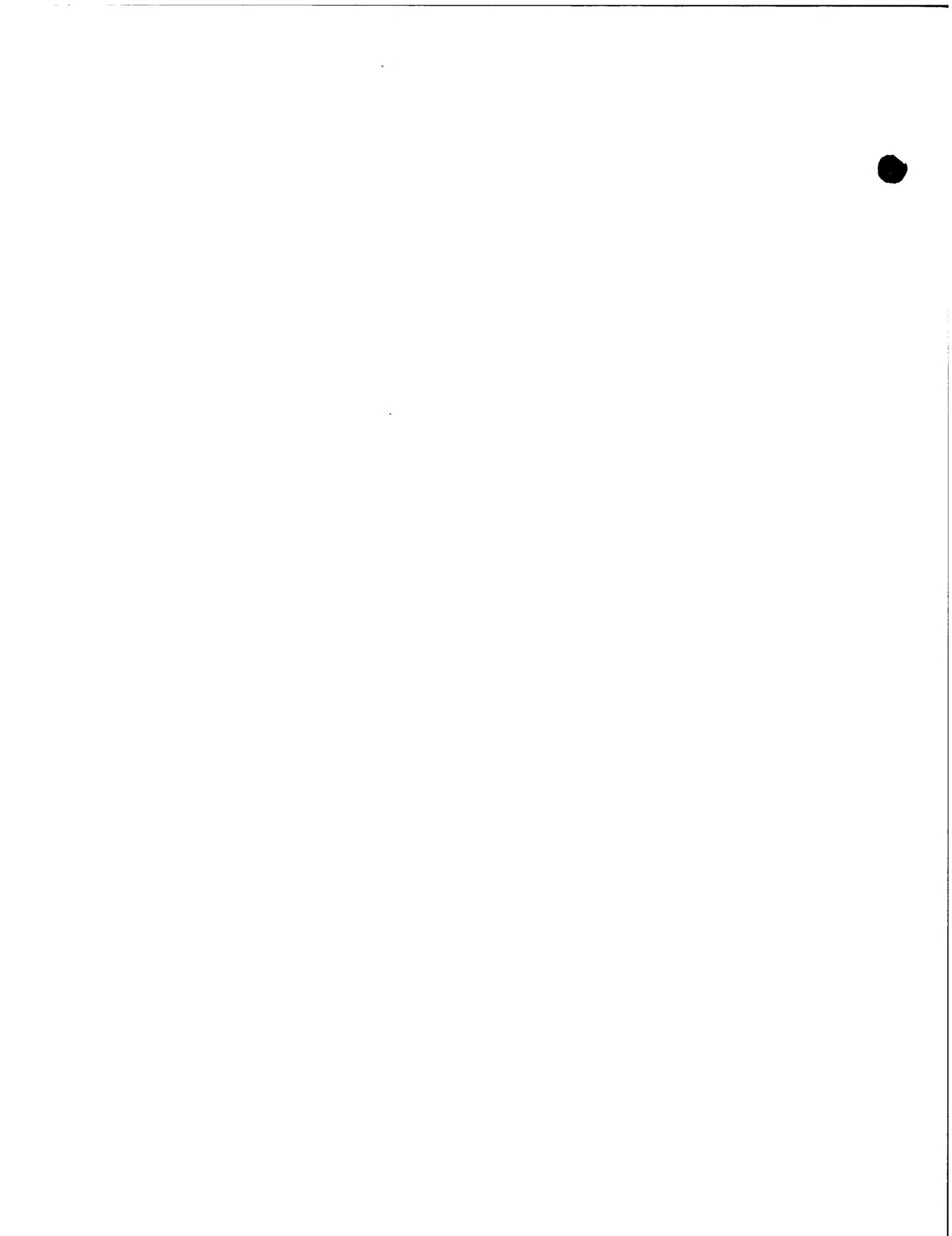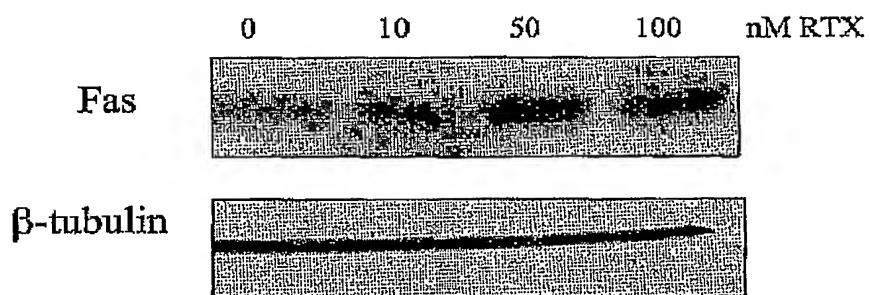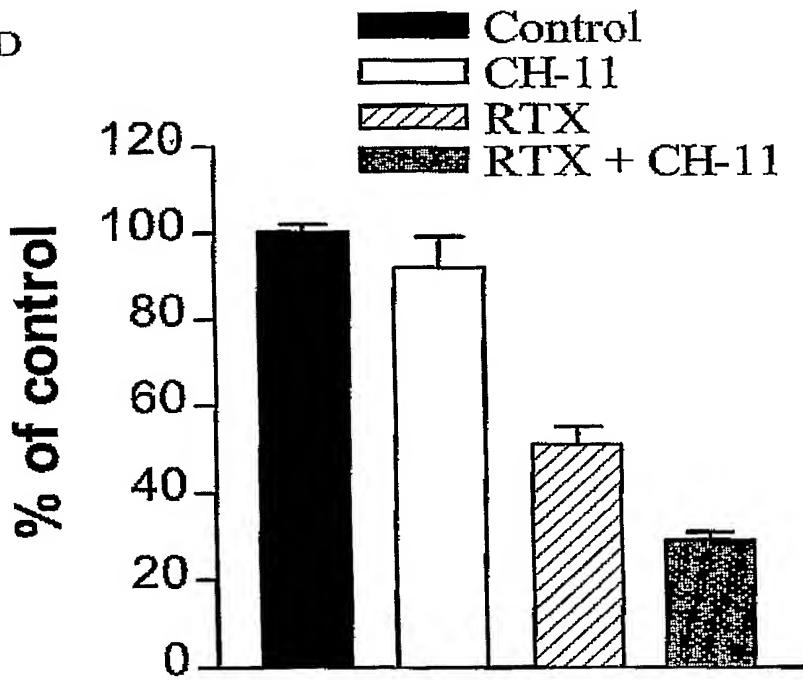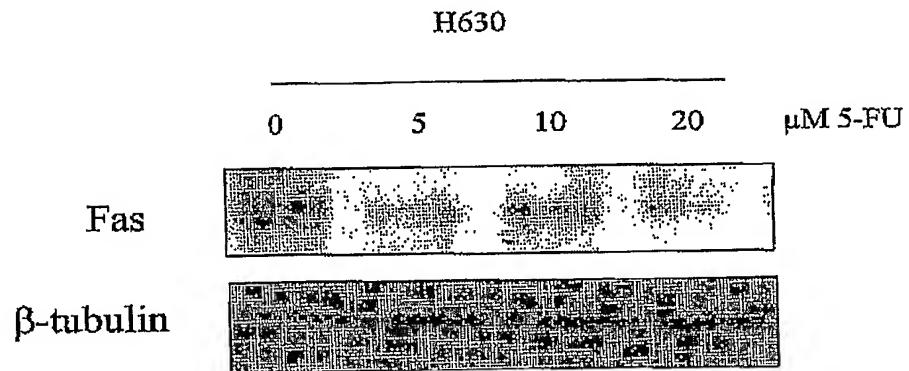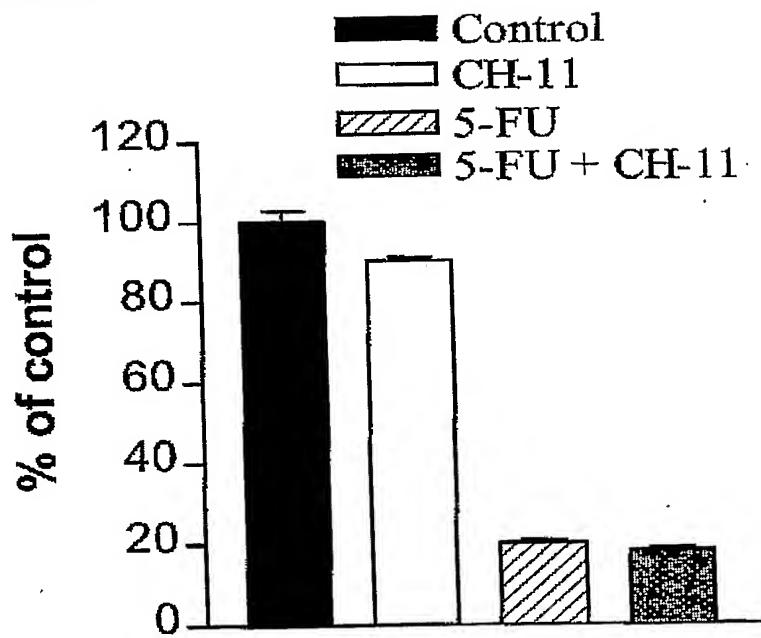
# 20/20
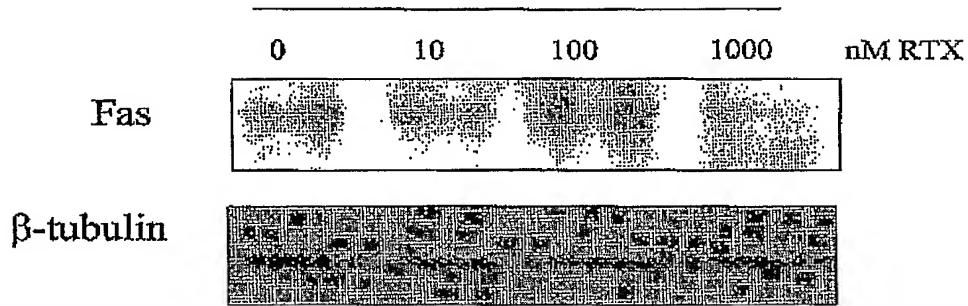
Figure 6G



Figure 6H

PCT/GB2004/005008